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# XXII. A COMPARATIVE INVESTIGATION OF THE CORRESPONDING PROTEINS OF COW AND OX SERUM, COW'S COLOSTRUM AND COW'S MILK BY THE METHOD OF PROTEIN RACEMISATION.

By HERBERT ERNEST WOODMAN.

*From the Institute for the Study of Animal Nutrition, School of Agriculture, Cambridge University.*

(*Received January 29th, 1921.*)

### Method of protein racemisation.

The method has its basis in the initial observations of Kossel [1911, 1912, 1913] and of Dakin [1912] that when solutions of protein in dilute sodium hydroxide solution are kept at 37°, they suffer a progressive diminution in the value of their optical rotatory power. Dakin [1912] attributed this change to a keto-enol tautomerism of the =CH—CO— groups in the protein complex and demonstrated further that the optical characters of the individual amino-acids obtained by hydrolysis of a racemised protein afforded some information as to their situation within the original protein molecule.

Dudley and Woodman [1915] were able to detect structural differences in the amino-acid make-up of the caseinogens of the cow and the sheep by racemising the proteins under comparable conditions, hydrolysing the racemised proteins and comparing the optical properties of the corresponding amino-acids. They were also able to confirm the identity of the euglobulin and pseudoglobulin of cow's colostrum [1918]. Dakin and Dale [1919] demonstrated in a similar manner the existence of structural differences between the albumins of the eggs of the duck and the hen, a result which was confirmed by subsequent biological trials.

### A simplified racemisation method.

The main objections which may be urged against the method of protein racemisation referred to above are twofold.

Firstly, like most other methods in protein research, the method involves certain tedious and laborious processes such as hydrolysis and the subsequent separation of the amino-acids by the Fischer-Levene esterification process and the Kossel-Kutscher method for the diamino-acids. Large amounts of material, often only prepared with difficulty, are requisite.

Secondly, the nature of the processes involved prevents the method from being quantitative, and differentiation between proteins is only possible when very sharp distinctions in the optical characters of corresponding amino-acids occur. In addition to this, certain of the amino-acids like proline may undergo partial racemisation during their isolation from the hydrolytic products of the racemised protein, and thus the significance of the optical findings in such cases may be obscured.

In the investigation to be described in the present communication, the racemisation reaction has been utilised for the same purpose in a much simpler manner by following quantitatively, and under perfectly definite conditions, the actual course of the racemisation of certain proteins and thus obtaining what might be termed their "racemisation curves." Preliminary trials carried out by Dudley and Woodman [1918] in their work on the globulins of colostrum had given rise to the hope that it might be possible to establish identity or non-identity of related proteins by such means.

If, for example, the fall in rotation of a 2 % solution of globulin in $N/2$ soda be followed at 37°, it is found that if the specific rotations be plotted against the time in hours during which the reaction has been allowed to proceed, then the readings fall on a perfectly smooth curve of the type shown in Diagram I. The specific rotation of the solution, which initially is about $-80°$, sinks very rapidly at first, then more slowly and subsequently after about 250 hours attains a practically constant value of about $-22°$. If $N/4$ alkali be used instead of $N/2$, the gradient of the curve is less steep. This behaviour is connected with the role played by the alkali in the reaction and this aspect of the question will be discussed in a further communication dealing with the mechanism of the reaction between proteins and dilute alkali at low temperatures. At present it need only be pointed out that the actual significance of the reaction does not materially affect the value of the results obtained by its use in comparing different proteins, since all the trials are carried out under strictly comparable conditions.

On the assumption that each individual protein will possess its own specific set of curves, the racemisation reaction can be made the basis of a method for testing identity or non-identity of proteins. If two proteins are to be pronounced structurally identical, then if racemised under the same conditions, their solutions must show the same initial rotation, the same final rotation and the same rate of diminution of optical rotation; the last named is probably intimately connected with the intramolecular structure of the protein. Not only this, the proteins must continue to display identical optical behaviour when the concentrations of the alkali and of the protein are varied. Thus the test can be made very exhaustive. If the two proteins are not structurally identical, this will be revealed by their possessing distinct sets of racemisation curves.

It must be pointed out, however, that the amount of difference between the optical values of two proteins in dilute alkali is not an absolute measure

of the amount of structural difference existing between them, since the optical rotatory power of a complex substance is by no means a simple additive function of the activities of its several constituents. All that the method can claim to do is to establish structural identity or non-identity, and in this respect it is perhaps superior to the van Slyke method, since it is manifestly possible for two proteins to be quantitatively identical with regard to their amino-acid content, and yet be distinct proteins by virtue of differences in the order of linkage of the amino-acids. The caseinogens of the cow and the sheep afford such an example; these proteins cannot be differentiated by the van Slyke method, but were shown to be different stereochemically by the ordinary protein racemisation method [Dudley and Woodman, 1915]. The van Slyke method and the simplified racemisation method might be most usefully employed in conjunction with each other.

The simplified racemisation method can claim the following advantages, that it is relatively simple and rapid and only requires the use of small amounts of material. It has been employed with complete success in investigating the relationships existing amongst the corresponding proteins of serum, colostrum and milk.

### The corresponding proteins of cow and ox serum, cow's colostrum and cow's milk.

An examination of the available data regarding the structural relationships which exist among these proteins serves to reveal the existence of discord between the chemical and biological evidence. The investigation of the structure of a protein by chemical means is manifestly a matter of extreme difficulty. It is possible to determine quite readily, and with a fair degree of accuracy, the *amounts* of the various amino-acids in any given protein by the van Slyke distribution method; but the study of the precise *order* in which those amino-acids are linked up within the protein molecule presents a baffling problem, even in the case of simple peptone derivatives.

It is scarcely a matter of surprise, then, that in the past, the chemist has had to rely largely on the biologist to furnish him with information regarding the relationships existing among any given class of proteins. The biologist has been able to pursue this line of enquiry with the help of certain well-known methods, such as the precipitin and anaphylaxis methods, which have been evolved by workers on immunity.

In this way he has been able to draw the following conclusions. Firstly, that the globulin of serum is identical with the globulin of colostrum. Secondly, that the albumins of serum, milk and colostrum are one and the same protein. Hamburger [1901], by the use of the precipitin method, demonstrated the existence of a very close relationship between the proteins of blood and milk. Further confirmation of this was derived from the work of Schlossmann and Moro [1903], who concluded that the albumin of the blood was identical with lactalbumin and because of this were inclined to the assumption that the

infant, in the early days of its existence, may be able to absorb directly the milk albumin. Bauer and Engel [1911], after testing the question by means of the method of complement deviation, also came to the conclusion that the serum proteins were identical with the corresponding proteins of milk and colostrum.

The identity of the proteins of serum and milk, as established by biological methods, has led physiologists to make the following generalisation: That it is not necessary to assume a distinct mammary synthesis for the elaboration of the albumin and globulin of the mammary secretion, but that these are present in the colostrum and milk as a result of a simple and direct transference from the blood stream.

The results of chemical investigation, however, are not wholly in harmony with the above findings. Several earlier workers, as a result of work based on simple chemical analysis and a study of the methods of precipitation, drew conclusions in accord with those obtained in the biological investigations. Such evidence, however, is of little use in drawing conclusions regarding the structure of the protein molecule.

With regard to the globulins, the chemical work amply confirms the results obtained by biologists in demonstrating the identity of lactoglobulin and serum globulin. Conclusive evidence was furnished by the work of Hartley [1914] on serum globulin and of Crowther and Raistrick [1916] on the globulin which can be isolated in large amounts from colostrum and in very small amounts from normal milk. These investigators analysed the proteins by means of the van Slyke method, and a comparison of their data fails to reveal any differences in the quantitative nature of the amino-acid make-up of the various globulins.

In respect of the albumins, however, chemical evidence is available which would suggest that lactalbumin and serum albumin, far from being identical, are two distinct proteins. As far back as 1885, Sebelien [1885] prepared lactalbumin and serum albumin by similar methods and found that whereas lactalbumin from milk or colostrum has a specific rotation in water of about $-37°$, that of serum albumin is about $-63°$. Later, Halliburton [1890] showed that lactalbumin differed from serum albumin in its behaviour on heat coagulation and with regard to its precipitability by neutral salts. Referring again to the work of Hartley and of Crowther and Raistrick, a comparison of their van Slyke data for the two albumins reveals marked differences in respect of their quantitative amino-acid content. Differences occurred in every item of the analyses, but were especially marked with regard to the basic nitrogenous constituents.

The present work, therefore, was undertaken partly with a view to investigating this discrepancy between the biological and chemical findings and partly with the object of testing the applicability of the simplified racemisation method to problems of this type.

It will be convenient at this stage to summarise the main results of the

investigation. Firstly, the euglobulin and pseudoglobulin fractions in both serum and colostrum have been shown to be identical proteins. Secondly, serum globulin has been found to be identical with colostrum globulin. Thirdly, as one would anticipate, no differences were detected between the corresponding proteins of cow and ox serum. Fourthly, the albumins of milk and colostrum were found to be the same individual protein. Fifthly, lactalbumin and serum albumin have been shown to be distinct chemical individuals and it is reasonable to speculate whether, by the use of the more modern and refined anaphylactic methods, it would not be possible to obtain evidence in harmony with the chemical results as to the non-identity of the two albumins.

If the chemical results are to be accepted, it must be assumed that a distinct mammary synthesis is necessary for lactalbumin as well as for caseinogen; and it would appear that the temporary power of the organism of permitting relatively large amounts of serum globulin to be transferred from the blood stream to the colostral secretion is of the nature of an emergency measure. From the results of Crowther and Raistrick [1916] it would further appear that even when normal lactation sets in, the serum globulin can leak, as it were, in extremely small amounts into the milk secretion. The basal nitrogenous constituents of normal milk, namely caseinogen and lactalbumin, are elaborated by the mammary gland both in the colostral and the normal lactation periods.

### Preparation of the proteins.

For the purposes of comparative work, it was essential to prepare the proteins from the different sources by the use of the same methods, since it was at least possible that the chemical composition of a protein preparation might vary within limits, if the method of isolation were varied. Experiments will be described later, however, in which it was found impossible to detect, by the use of the simplified racemisation method, differences between two samples of globulin, which had been prepared by two absolutely different methods.

The total globulin was removed from neutral solution by half-saturation with ammonium sulphate; the pseudoglobulin and euglobulin were separated by dialysis. The albumin was obtained from the filtrate from the total globulin by the addition of the requisite volume of $N/5$ sulphuric acid together with an equal volume of saturated ammonium sulphate solution, in order to maintain half-saturation. The albumin was freed from ammonium sulphate by dialysis.

The samples of protein relied on had all been fractionally precipitated seven times, and in order to be sure that further precipitation would not affect the optical behaviour of the protein in dilute alkali, portions of protein from successive fractionations (namely, the sixth and the seventh) were racemised separately. In this way, it was possible to obtain a check on the completeness of the separation of the albumin from the globulin.

*Proteins of ox serum.*

*Globulin.* Fresh ox blood was defibrinated and centrifuged without delay. After filtering through cotton wool, the serum was diluted with its own volume of water and an equal volume of saturated ammonium sulphate solution was added. After standing several hours, the precipitate of globulin was filtered off on a large Buchner funnel and the filtrate was used for the isolation of the albumin. The crude globulin was redissolved in distilled water and the bulk made up to that of the original diluted serum. After filtering the solution, an equal volume of saturated ammonium sulphate solution was again added and the precipitated globulin was filtered off. The precipitation of the globulin was carried out in all seven times; a small portion of the precipitate after the sixth fractionation, however, was retained for comparison with the globulin obtained at the seventh precipitation. The final solution of globulin was filtered and dialysed in large conical shaped parchment dialysers (which had previously been immersed in a 5 % solution of gelatin and then submitted to the action of formalin vapour). After dialysing for several days in running tap water, the process was continued in frequent relays of distilled water, until the solution was free from sulphate. In this manner, the globulin was separated into pseudoglobulin, which remained in solution, and euglobulin, which separated out during dialysis.

The euglobulin was filtered off from the pseudoglobulin and was dissolved in the minimum amount of 0·6 % NaCl solution. After filtering the solution, the euglobulin was reprecipitated by the addition of about fifteen times its volume of distilled water. The precipitate settled on standing and the bulk of the water was decanted off. After filtering on to a Buchner funnel, the euglobulin was washed with distilled water and finally dried with alcohol and anhydrous ether.

The filtered pseudoglobulin solution was cooled in ice and the protein precipitated by the addition of ice cold alcohol. The precipitate was filtered off and dried with alcohol and anhydrous ether. The amount of pseudoglobulin obtained in this process was appreciably greater than that of the euglobulin.

Two per cent. solutions of pseudoglobulin obtained as a result of six and seven fractionations respectively were racemised by means of $N/4$ NaOH. The following readings are taken from the series of polarimetric determinations made on the solutions from time to time during the course of the racemisation.

| Pseudoglobulin after 6 precipitations 2 % in $N/4$ NaOH | | Pseudoglobulin after 7 precipitations 2 % in $N/4$ NaOH | |
|---|---|---|---|
| Time in hours | Specific rotation | Time in hours | Specific rotation |
| 1½ | −75·5° | 1½ | −76·0° |
| 26 | −54·5 | 24 | −55·0 |
| 48 | −48·5 | 53 | −48·0 |
| 96 | −42·0 | 93 | −42·0 |
| 144 | −38·0 | 140 | −38·0 |

Similar agreement was obtained when 2 % solutions of these samples in $N/2$ alkali were racemised and from this it was concluded that after six precipitations a definite and individual globulin preparation had been isolated, whose composition and properties were unaffected by further fractionation.

*Albumin.* To the filtrate from the total globulin was added the requisite volume of $N/5$ sulphuric acid to effect precipitation of the albumin, the necessary amount for complete precipitation being previously determined on a small bulk of solution. After allowing to stand for 24 hours, the albumin was filtered on to a large Buchner funnel. It was then redissolved in distilled water and made exactly neutral by means of very dilute caustic soda; the solution was filtered, made up to the original volume and to it was added an equal volume of saturated ammonium sulphate solution. The small amount of globulin which separated was filtered off and added to the bulk of crude globulin. The albumin was precipitated from the filtrate by once more acidifying with the requisite amount of $N/5$ sulphuric acid. This process of fractionation was carried out in all seven times. After the fifth fractionation, no globulin precipitate was obtained when ammonium sulphate was added to the neutral solution.

The final albumin precipitate was dissolved in water after being well washed with saturated ammonium sulphate solution, and the filtered solution was submitted to exhaustive dialysis until free from sulphate. The dialysed solution was filtered and cooled in ice, and the albumin was precipitated with ice cold alcohol. It was subsequently dried by means of alcohol and anhydrous ether and was obtained as a white powder, soluble in water.

It was found, as with the globulin, that samples of albumin from the sixth and seventh fractionations displayed identical optical behaviour in $N/2$ and $N/4$ caustic soda.

*Proteins of cow serum.*

The globulins and albumin were isolated from fresh cow's blood by exactly the same methods used for the preparation of the corresponding proteins from ox blood. The preparations were the result of seven fractional precipitations. As with ox serum, the amount of pseudoglobulin obtained from the cow serum was very much greater than that of euglobulin.

*Proteins of colostrum.*

For the purposes of this investigation, the first drawn milk from two cows after parturition was collected and mixed. The colostrum was diluted with twice its bulk of distilled water and the caseinogen was precipitated by the addition, drop by drop, of 50 % acetic acid until complete precipitation had occurred. During the acidification, the bulk of liquid was vigorously stirred in a tall glass jar. The caseinogen was allowed to settle and the clear supernatant liquid was siphoned off; the residual precipitate of caseinogen was filtered off and the filtrate was added to the bulk of the solution of globulin

and albumin. The latter was then filtered, made exactly neutral with dilute caustic soda and the proteins were isolated by precisely the same methods as have been described for the separation of the serum proteins. They were obtained as white powders and had all been fractionally precipitated seven times. The colostrum pseudoglobulin was found to preponderate considerably in amount over the euglobulin.

*The albumin of milk.*

The isolation of globulin from milk was not attempted. Mixed milk from several cows was diluted with twice its bulk of distilled water and the caseinogen was separated in the manner already described for colostrum. The subsequent isolation of the lactalbumin was carried out by the method used for the serum and colostrum albumins. It was purified by seven fractionations, and after drying with alcohol and anhydrous ether, gave a white voluminous powder completely soluble in water. Tests carried out on small portions of albumin from the sixth and seventh fractionations showed no difference in optical behaviour with either $N/2$ or $N/4$ NaOH.

DETAILS OF METHOD FOR FOLLOWING COURSE OF RACEMISATION OF PROTEINS IN DILUTE ALKALI AT 37°.

Before investigating the behaviour of the proteins in dilute alkali, the samples were finely ground up and then dried *in vacuo* over calcium chloride for several days. The determinations were carried out in the following manner. Exactly 1 g. of dried protein was weighed out into a 50 cc. flask containing a little distilled water. The flask was then shaken gently to bring the protein into solution or suspension. 25 cc. of $N$ NaOH free from carbonate (or $N/2$ as the case may be) were then run in from a pipette, and after mixing gently, the volume was made up almost to the 50 cc. mark with distilled water. The flask was then placed in the incubator at 37° and after the contents had become warm, the volume was made up exactly to the 50 cc. mark. After a short time, the solution was filtered quickly into a small dry flask, which was then stoppered and kept in the incubator. From time to time, determinations of the optical rotation were made in a 1 d. polarimetric tube, using a Schmidt and Haensch instrument and sodium light. This procedure was continued for about 300 hours, when the value had become practically constant.

Graphs were then constructed showing the progress of racemisation. The ordinates represented specific rotation values and the abscissae the number of hours during which the reaction had been allowed to proceed.

It was found inadvisable to attempt to dissolve the proteins directly in the dilute alkali, as the substances show a tendency to become coated with glutinous material, which retards the dissolving process. The experience of this investigation showed that in work of this type it would be more convenient not to precipitate the proteins from solution by means of alcohol,

but to deal with solutions of protein which had been concentrated considerably *in vacuo* at low temperature. With preparations of this kind, not the slightest difficulty is experienced in readily making up solutions of known concentration in alkali.

Such perfectly clear and only very slightly yellow solutions of protein in the dilute alkali ($N/2$ and $N/4$ NaOH) have been preserved over periods of months without the slightest indications of bacterial decomposition.

The investigation of each sample of protein was carried out at least in duplicate; the duplicate sets of readings showed very satisfactory agreement. In most cases, however, three and even four series of optical readings were taken in order to determine the positions of the various racemisation curves with the fullest possible degree of certainty. The experimental error in reading with a Schmidt-Haensch instrument is very small, and with practice the reading in a 1 d. tube could be determined to 0·01°. Thus the specific rotation of a 2 % solution of protein could be relied on to 0·5°.

### Racemisation of the globulins.

The figures obtained in the racemisation of the various globulins are given in Table I and Diagram I. The data in each case represent the progress of the racemisation up to about 250 hours, when the value of the specific rotation had attained a practically constant value. After this, however, the rotation still underwent a very small, but quite definite diminution in value. Discussion of this point, however, will be left to a future communication.

Table I. *Figures obtained in the racemisation of the various globulins.* (*The observations for the pseudoglobulins of cow serum and colostrum are given in Diagram I.*)

| 1 Ox serum pseudoglobulin. 2 % in $N/2$ NaOH | | 2 Cow serum euglobulin. 2% in $N/2$ NaOH | | 3 Ox serum euglobulin. 2 % in $N/2$ NaOH | | 4 Cow colostrum euglobulin. 2 % in $N/2$ NaOH | |
|---|---|---|---|---|---|---|---|
| Time in hours | Specific rotation | Time in hours | Specific rotation | Time in hours | Specific rotation | Time in hours | Specific rotation |
| 1½ | −74·5° | 1½ | −74·0° | 2 | −72·5° | 1 | −75·5° |
| 22 | −48·0 | 24 | −47·5 | 7 | −62·0 | 25 | −46·5 |
| 49 | −37·0 | 49 | −37·0 | 24 | −47·0 | 48 | −37·0 |
| 67½ | −33·0 | 73 | −32·0 | 48 | −37·5 | 74 | −32·0 |
| 117½ | −27·5 | 102 | −29·0 | 72 | −32·0 | 96 | −30·0 |
| 173 | −24·5 | 126 | −27·0 | 98 | −29·5 | 125 | −27·0 |
| 242 | −22·0 | 146 | −25·5 | 120 | −27·5 | 147 | −25·5 |
| | | 198 | −24·0 | 146 | −25·5 | 198 | −23·5 |
| | | 240 | −22·0 | 175 | −24·5 | 242 | −22·0 |
| | | | | 218 | −23·0 | | |

| 5 Ox serum pseudoglobulin. 2 % in $N/4$ NaOH | | 6 Cow serum euglobulin. 2 % in $N/4$ NaOH | | 7 Ox serum euglobulin. 2 % in $N/4$ NaOH | | 8 Cow colostrum euglobulin. 2 % in $N/4$ NaOH | |
|---|---|---|---|---|---|---|---|
| 1½ | −75·5° | 2 | −74·5° | 1½ | −76·0° | 2 | −74·0° |
| 24 | −55·0 | 7 | −66·5 | 24 | −55·0 | 6 | −67·0 |
| 48 | −48·5 | 24 | −54·5 | 50 | −48·0 | 25 | −54·0 |
| 72 | −45·0 | 48 | −48·0 | 72 | −45·5 | 50 | −48·0 |
| 96 | −43·0 | 78 | −44·5 | 96 | −42·0 | 73 | −45·0 |
| 144 | −38·0 | 97 | −42·5 | 144 | −38·0 | 96 | −42·0 |
| 189 | −35·0 | 145 | −38·0 | 197 | −34·5 | 146 | −38·0 |
| 240 | −33·5 | 192 | −35·0 | 240 | −33·5 | 196 | −35·0 |
| | | 240 | −34·0 | | | 242 | −33·0 |

*Comments on Table I.*

In order to interpret more easily the different racemisation data, it is advantageous to represent the course of the racemisations graphically. Only one such graphical representation is given here for the pseudoglobulins (Diagram I). From a study of it, the following general features may be noted. The initial specific rotation of a 2 % pseudoglobulin solution in alkali is about $-80°$; this factor diminishes rapidly in the first stages of the racemisation and ultimately attains a practically constant value of about $-22°$. The curves illustrate clearly the different effects of $N/2$ and $N/4$ alkali. Though the strength of the alkali does not appear to affect appreciably the initial rotations of the solutions, yet the rate of fall of rotation is slower in the weaker alkali.

1. *Comparison of cow and ox serum globulins.*

The figures obtained for the euglobulin and pseudoglobulin of cow and ox serum demonstrate the identical optical behaviour displayed by these proteins not only in $N/2$ but also in $N/4$ NaOH. Such a result is of course in keeping with the presumed structural identity of corresponding proteins from different members of the same species, irrespective of sex. The determinations, however, afforded a check not only on the correctness of the method of working, but also on the chemical individuality of serum globulin prepared from different sources.

2. *Comparison of euglobulin and pseudoglobulin.*

The figures in Table I and Diagram I show that euglobulin and pseudoglobulin, whether from serum or from colostrum, display the same optical behaviour in $N/2$ as well as in $N/4$ alkali. This result, which harmonises with the assumption of the structural identity of euglobulin and pseudoglobulin, is confirmatory of the work of Miss Chick [1914], Hartley [1914], Crowther and Raistrick [1916], Dudley and Woodman [1918]. If, as Miss Chick postulated, the euglobulin is associated with traces of a lipoid substance, then the latter does not appear to have been present in sufficient amount in the samples investigated to affect their specific rotations in alkaline solutions to a measurable degree.

3. *Comparison of globulins of serum and colostrum.*

That the optical rotatory values of the globulins of serum and of colostrum are identical in $N/2$ and $N/4$ NaOH is shown by Diagram I, in which the data for the pseudoglobulins from the two sources have been plotted.

It will be seen that under each set of conditions, one curve can be drawn to satisfy equally the two sets of readings determined for the colostrum and the serum pseudoglobulin. Such slight discrepancies as may exist fall within the general error of experiment and this identity of optical behaviour may be taken as confirming the structural identity of the globulins of colostrum and of serum, a result which is in accord with the earlier chemical and biological work.

### Racemisation of the albumins.

The figures obtained in the racemisation of the various albumins are given in Table II and Diagram II.

Table II. *Figures obtained in the racemisation of the various albumins. (The observations for the albumins of cow colostrum and ox serum in N/4 NaOH are given in Diagram II.)*

| 1 Cow serum albumin 2% in N/2 NaOH | | 2 Ox serum albumin 2% in N/2 NaOH | | 3 Cow colostrum albumin 2% in N/2 NaOH | |
|---|---|---|---|---|---|
| Time in hours | Specific rotation | Time in hours | Specific rotation | Time in hours | Specific rotation |
| 1 | −70·0° | 1 | −69·5° | 1 | −85·0° |
| 24 | −47·5 | 2 | −67·0 | 2 | −77·5 |
| 44 | −39·5 | 7 | −59·5 | 6 | −68·5 |
| 73 | −33·5 | 24½ | −47·5 | 24 | −54·5 |
| 96 | −30·5 | 49 | −38·5 | 48½ | −44·5 |
| 120 | −27·5 | 73 | −34·0 | 73 | −38·0 |
| 146 | −25·0 | 98 | −30·5 | 96½ | −34·0 |
| 168 | −22·5 | 121 | −28·0 | 121½ | −31·0 |
| 213 | −19·5 | 149 | −24·5 | 145 | −28·5 |
| 240 | −18·0 | 169 | −23·0 | 168 | −26·0 |
| | | 192 | −21·0 | 192 | −24·0 |
| | | 218 | −19·0 | 217 | −23·0 |
| | | 242 | −18·0 | 240 | −22·0 |

| 4 Milk albumin 2 % in N/2 NaOH | | 5 Cow serum albumin 2 % in N/4 NaOH | | 6 Milk albumin 2 % in N/4 NaOH | |
|---|---|---|---|---|---|
| Time in hours | Specific rotation | Time in hours | Specific rotation | Time in hours | Specific rotation |
| 1 | −85·5° | 1 | −74·0° | 1 | −89·0° |
| 4½ | −70·5 | 25 | −57·5 | 6 | −79·5 |
| 24 | −55·0 | 43 | −53·0 | 23 | −66·0 |
| 50 | −44·0 | 67 | −47·5 | 48 | −58·5 |
| 72 | −38·5 | 91 | −44·5 | 68 | −54·0 |
| 97 | −34·0 | 125 | −39·5 | 96 | −49·0 |
| 120½ | −31·0 | 144 | −38·0 | 125 | −45·5 |
| 149 | −27·5 | 191 | −34·0 | 140 | −44·0 |
| 173 | −26·0 | 261 | −31·0 | 169 | −41·5 |
| 193 | −24·0 | | | 192 | −38·5 |
| 240 | −21·5 | | | 221 | −37·0 |
| | | | | 250 | −35·5 |

*Comments on Table II.*

A comparison of the figures given in Tables I and II and Diagrams I and II demonstrates the distinct chemical individuality of the globulin and albumin fractions both in serum and in colostrum.

In carrying out the racemisation trials with the albumin preparations, it was noticed that in every case a small amount of sediment separated out in the first few hours from the alkaline solutions. This was readily removed by filtration, a perfectly clear solution remaining.

In all cases, the course of racemisation could be represented graphically by a perfectly smooth curve of the type already described. As with the globulins, the action of $N/4$ soda occasioned a slower rate of diminution of optical rotation than was the case when $N/2$ alkali was employed.

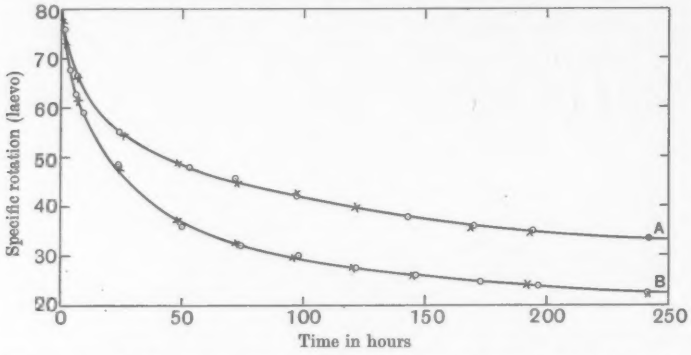


Diagram I. Showing identical optical behaviour of Cow Colostrum Pseudoglobulin [ × ] and Cow Serum Pseudoglobulin [ ⊙ ] in $N/2$ and $N/4$ NaOH.

Curve A. 2 % solutions of protein in $N/4$ NaOH.
Points denoted by × colostrum pseudoglobulin.
" " ⊙ serum pseudoglobulin.
Curve B. 2 % solutions of protein in $N/2$ NaOH.
Points denoted by × colostrum pseudoglobulin.
" " ⊙ serum pseudoglobulin.

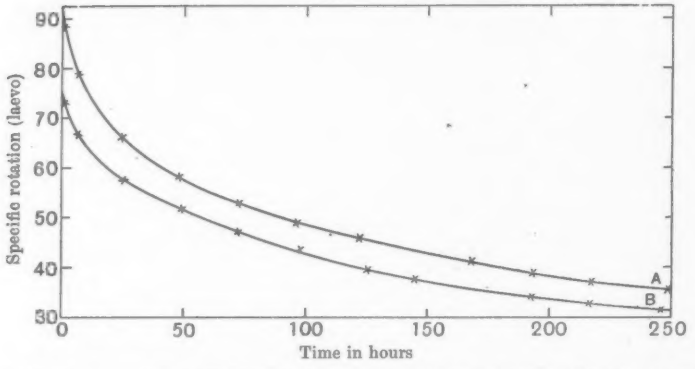


Diagram II. Showing different optical behaviour of Cow Colostrum Albumin (A) and Ox Serum Albumin (B) in $N/4$ NaOH.

Curve A. 2 % cow colostrum albumin in $N/4$ NaOH.
Curve B. 2 % ox serum albumin in $N/4$ NaOH.

1. *Comparison of cow and ox serum albumins.*

The results tabulated in Table II (1, 2, 5) and curve B, Diagram II, are conclusive as to the structural identity of the albumins of cow and ox serum.

### 2. *Comparison of the lactalbumins of colostrum and milk.*

The figures in columns 3, 4, and 6 of Table II and curve A, Diagram II show that colostrum and milk albumins display identical optical behaviour when incubated under comparable conditions with $N/2$ and $N/4$ soda. This is indicative of their structural identity and is in agreement with the results of biological tests and the work of Crowther and Raistrick [1916] on the van Slyke analysis of these proteins.

### 3. *Comparison of serum albumin and lactalbumin.*

The readings obtained in the racemisation of lactalbumin and serum albumin reveal marked differences between the optical behaviour of these proteins not only in $N/2$, but also in $N/4$ alkali. This difference is manifest in the initial rotations displayed in alkali, for whereas the specific rotation of serum albumin is initially about $-75°$, that of lactalbumin is about $-92°$ (taken from curves). A comparison of these data with the specific rotations for these proteins determined by Sebelien [1885] in aqueous solution shows a striking difference in the manner in which the nature of the solvent affects the rotation of the two proteins.

| | Serum albumin | Lactalbumin |
|---|---|---|
| $[\alpha]_D$ in water ... ... | $-63°$ | $-37°$ |
| $[\alpha]_D$ in $N/4$ NaOH ... | $-75°$ | $-92°$ |

These figures bring out very clearly the existence of structural dissimilarity between the two albumins. There can be little doubt that serum albumin and lactalbumin are two distinct proteins, though it is difficult to understand why the biological tests have failed to reveal this fact.

A study of Diagram II (which shows graphically the courses of racemisation in $N/4$ NaOH) reveals a remarkable parallelism between the curves of the two albumins.

The same feature is noticed if the curves in $N/2$ NaOH be drawn and compared. It may be that the structural differences existing between the two albumins are only of an inconsiderable character and that lactalbumin is actually formed from serum albumin as a result of comparatively slight changes brought about by the activity of the mammary gland. This speculation is suggestive of the possibility of a simple transformation of serum albumin into lactalbumin, or *vice-versa*.

### Racemisation data of heat-coagulated pseudoglobulin.

A sample of colostrum pseudoglobulin was coagulated by raising the temperature of its solution in water gradually to 90°. The coagulated protein was filtered off, washed successively with alcohol and ether and dried in the steam oven. 2 % solutions in $N/2$ and $N/4$ NaOH gave the following readings on racemising at 37°.

| In $N/2$ NaOH | | In $N/4$ NaOH | |
|---|---|---|---|
| Time in hours | Specific rotation | Time in hours | Specific rotation |
| 1 | −76·0° | 2 | −75·0° |
| 4½ | −66·0 | 24 | −55·0 |
| 24 | −47·5 | 48 | −48·5 |
| 48 | −38·0 | 72 | −45·0 |
| 72 | −32·5 | 120 | −39·5 |
| 120 | −27·0 | 165 | −36·0 |
| 169 | −24·5 | 216 | −34·0 |
| 220 | −22·5 | | |

A comparison of these data with those plotted in Diagram I shows clearly that heat coagulation of the protein had not in any way affected its optical rotatory values in alkali.

### Racemisation of samples of colostrum globulin prepared by absolutely different methods.

The writer had at his disposal, owing to the kindness of Drs. Crowther and Raistrick, a sample of colostrum pseudoglobulin which had been investigated by them in their comparative work already referred to. This had been prepared from colostrum by removing the caseinogen with saturated potash alum solution, and saturating the neutralised filtrate with anhydrous magnesium sulphate to obtain the globulin. The crude globulin was then purified by repeated salting out from solution with $MgSO_4$ and finally dialysed, the pseudoglobulin being subsequently thrown out of solution with alcohol. 2 % solutions of this preparation in $N/2$ and $N/4$ NaOH were investigated at 37° with the following results.

| In $N/2$ NaOH | | In $N/4$ NaOH | |
|---|---|---|---|
| Time in hours | Specific rotation | Time in hours | Specific rotation |
| 2 | −73·0° | 2½ | −73·0° |
| 24 | −47·5 | 24 | −55·0 |
| 48 | −37·5 | 49 | −48·0 |
| 74 | −31·5 | 73½ | −44·5 |
| 96 | −29·5 | 97 | −41·5 |
| 144 | −26·0 | 145 | −38·0 |
| 196 | −23·5 | 198 | −35·0 |
| 240 | −22·0 | 240 | −33·5 |

A comparison of these figures with those enumerated in Table I and Diagram I shows that the samples of globulin prepared by two different methods displayed identical optical behaviour in $N/2$ and $N/4$ alkali. This result supports the view that globulin is a definite chemical individual, whose composition is unaffected by its method of preparation.

SUMMARY.

1. A simple optical method has been described whereby it is possible to establish identity or non-identity of related proteins.

2. Evidence of the identity of euglobulin and pseudoglobulin, whether from serum or colostrum, has been brought forward.

3. The identity of the globulins of cow and ox serum and of colostrum has been confirmed.

4. The albumin of milk has been shown to be identical with the albumin of colostrum.

5. Lactalbumin and serum albumin have been found to be two distinct proteins. From this, the conclusion is drawn that a distinct mammary synthesis is necessary for lactalbumin as well as for caseinogen.

6. The optical rotatory properties of pseudoglobulin in alkali have been shown to be unaffected by heat coagulation.

7. The corresponding proteins of cow and ox serum have been compared and found identical.

8. Evidence has been adduced in support of the view that globulin is a definite chemical individual, whose composition is unaffected by its mode of preparation.

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# XXIII. ON THE THEORY OF THE BARCROFT DIFFERENTIAL BLOOD-GAS APPARATUS.

By THOMAS RICHARD PARSONS (*Michael Foster Student*).

(*From the Physiological Laboratory, Cambridge.*)

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*Report to the Medical Research Council.*

During the course of a lengthy series of investigations in which the Barcroft differential apparatus has been used for blood-gas measurements a certain number of results were encountered which seemed to suggest that the evolution of a given volume of gas in one of the bottles of the apparatus might give rise to a somewhat smaller difference of level on the clove oil manometer than the absorption of an equal volume of the gas from the same bottle under similar conditions. In other words, it seemed possible that the value of the constant of the apparatus might not be identical for the two cases of evolution and absorption of gas. At first sight there seems to be some *a priori* reason why such a difference should be expected, for in the ordinary method of using the apparatus the air in each bottle is always at atmospheric pressure at the beginning of the experiment; if then the apparatus is closed and an evolution of gas takes place in one bottle the pressure in each bottle will be increased above atmospheric, while in the case where an absorption of gas occurs, the pressure in each bottle will sink below atmospheric. These relationships have been pointed out by Hill [1915], and more recently by Münzer and Neumann [1917]: we mention them here in order to emphasise the circumstance that when an evolution of gas is taking place the conditions in the apparatus are somewhat different from those obtaining during gas absorption. As no data appear to exist in the literature from which it could be determined whether or not this difference of pressure conditions would produce an appreciable effect on the results obtained by the apparatus, and in view of the widespread use of the apparatus, particularly for the determination of the percentage saturation of blood with oxygen by comparing the further amount of oxygen the blood sample is capable of absorbing from the air in the apparatus with the total amount of oxygen subsequently expelled from the fully saturated blood by treatment with potassium ferricyanide, it was decided that the matter was of sufficient importance for an experimental investigation.

While the work was in progress, a paper by Wertheimer [1920] became accessible dealing with the question of the extent to which the volume of gas evolved in one bottle of the apparatus (deduced from the resulting difference

of level on the manometer and the dimensions of the apparatus) should be corrected for the vapour tension of the water with which the gas is saturated. This consideration does not affect the method of treatment here given, as the actual volumes of air added or withdrawn have been measured directly, but it is necessary to call attention to Wertheimer's paper in order to complete the list of the more recent papers on the differential apparatus.

The apparatus used for the purpose was of the newest form described by Barcroft [1914]. In order to simplify the measurements and also to carry out the addition and abstraction of a measured volume of air from the bottle under as nearly similar circumstances as possible, the modification of Hoffmann's [1913] method of calibration described by Münzer and Neumann was chosen in preference to a chemical method. Minute details as to apparatus and procedure are unnecessary as they are contained in the accounts given by these authors. Suffice it to say that in order to imitate the actual conditions under which the differential apparatus is used, 3 cc. of distilled water were placed in each bottle, and the apparatus was hung over the side of a water bath in the usual way. By raising or lowering a reservoir we were able to expel into or abstract from one of the bottles a known volume (measured at the atmospheric pressure and at the temperature of the water bath) of the air collected over water in a graduated 1 cc. pipette, and at the same time to notice the difference of level produced on the clove oil manometer. In the first experiments which were carried out, the measuring pipette was filled completely with air, and by pressing successive quantities of this into the blood-gas bottle a series of readings was obtained in all of which the pressure in the apparatus was above atmospheric. The measuring pipette was then filled with water, and into it were drawn successive measured quantities of air from the bottle in order to obtain a series of readings in which the pressure was less than atmospheric. The results obtained in this way are recorded in Table I, in which a + sign indicates that air was forced into the bottle and a − sign that air was withdrawn from it. The values of the constant of the apparatus are calculated in terms of mm.$^3$ of gas per mm. difference of level of the manometer. It will be seen that the average value of the constant obtained by adding air to the apparatus is almost 4 % higher than the average value obtained in the experiments in which air was withdrawn. But a closer examination of these figures shows that in the experiments carried out at increased pressure a different portion of the air-measuring pipette was in use from that into which air was drawn in the experiments at diminished pressure. As the pipette used had not been specially calibrated it seemed possible that a difference in the accuracy of these two parts of the scale of graduations might partly account for this result. Further, it is evident that the error due to drainage of the water from the internal walls of the pipette will affect differently the readings taken under the two conditions. In all cases the volume of the water adhering to the sides of the pipette will cause an error such that the actual volume of air driven into or removed from the apparatus will be less than the apparent

Table I. *Preliminary experiments. Apparatus L.*

| Initial reading of air pipette cc. | Final reading of air pipette cc. | Vol. of air added or abstracted (room temp. and pressure) cc. | Difference of level in clove oil manometer cm. | Constant (cmm. gas per mm. difference of level) |
|---|---|---|---|---|
| ·353 | ·254 | +·099 | 3·16 | 3·13 |
| | ·212 | +·141 | 4·51 | 3·13 |
| | ·146 | +·207 | 6·59 | 3·14 |
| | ·092 | +·261 | 8·31 | 3·14 |
| ·389 | ·260 | +·129 | 4·19 | 3·08 |
| | ·183 | +·206 | 6·60 | 3·12 |
| | ·072 | +·317 | 10·04 | 3·13 |
| Mean value of constant for addition of gas | | | ... ... ... | 3·12 |
| ·044 | ·086 | −·042 | 1·45 | 2·90 |
| | ·121 | −·077 | 2·60 | 3·00 |
| | ·158 | −·114 | 3·71 | 3·07 |
| | ·160 | −·116 | 3·86 | 3·01 |
| ·002 | ·058 | −·056 | 1·87 | 3·00 |
| | ·093 | −·091 | 3·01 | 3·02 |
| | ·136 | −·134 | 4·33 | 3·09 |
| Mean value of constant for abstraction of gas | | | ... ... ... | 3·01 |

volume read off from the pipette, so that the values obtained for the constant of the apparatus, *i.e.* for the ratio $\frac{\text{volume of air added or withdrawn}}{\text{difference of level produced}}$, will throughout tend to be too large. But in the experiments in which air was added to the bottle, the lower part of the pipette was in use, and this would tend to contain an extra amount of drainage from the upper parts, so that the drainage error would be particularly large in this case. On the other hand, while air was being drawn out of the bottle only the upper portion of the pipette was in use, so that the drainage error would be less in accordance with the diminution in the amount of surface exposed. In view of these considerations, in later experiments, the "positive" and "negative" experiments were carried out alternately and in pairs, and the determinations were so arranged that as nearly as possible the same particular portion of the pipette was used for (say) a "positive" experiment as for the "negative" experiment which immediately succeeded it. In this way, although we were no more certain of the absolute volume of air measured, we ensured that this volume was measured under the same conditions and in the same portion of the pipette in both the "positive" and the "negative" experiment of each pair. Typical results obtained in this way are recorded in Table II from which it will at once be seen that the value of the constant obtained when the apparatus was working at increased pressure is identical, within the range of our measurements, with that observed when the general pressure in the apparatus was diminished.

In order to confirm the assumption that in these later experiments the measurement of the volume of air added to or withdrawn from the apparatus was not affected by an error due to the drainage of the confining liquid, a further series of observations was also carried out in which the measured volume of air was collected over mercury. It was found that the use of mercury

Table II. *Later experiments. "Positive" and "negative" determinations carried out alternately. Apparatus* 3 *and* 4.

| Initial reading of air pipette cc. | Final reading of air pipette cc. | Vol. of air added or abstracted (room temp. and pressure cc. | Difference of level in clove oil manometer cm. | Constant (cmm. gas per mm. difference of level) |
|---|---|---|---|---|
| ·035 | ·254 | −·219 | 6·54 | 3·35 |
| ·253 | ·023 | +·230 | 6·78 | 3·39 |
| ·053 | ·258 | −·205 | 6·21 | 3·30 |
| ·257 | ·057 | +·200 | 6·08 | 3·29 |
| ·034 | ·250 | −·216 | 6·57 | 3·29 |
| ·254 | ·036 | +·218 | 6·57 | 3·32 |
| ·047 | ·240 | −·193 | 5·84 | 3·31 |
| ·234 | ·047 | +·187 | 5·61 | 3·33 |
| ·029 | ·245 | −·216 | 6·34 | 3·41 |
| ·244 | ·033 | +·211 | 6·37 | 3·31 |
| ·034 | ·240 | −·206 | 6·17 | 3·34 |
| ·231 | ·040 | +·191 | 5·76 | 3·32 |
| ·013 | ·235 | −·222 | 6·61 | 3·36 |
| ·224 | ·016 | +·208 | 6·23 | 3·34 |
| ·013 | ·123 | −·110 | 3·36 | 3·27 |
| ·124 | ·014 | +·110 | 3·35 | 3·28 |
| Mean constant for addition of gas ... ... ... ... ... | | | | 3·32 |
| Mean constant for abstraction of gas ... ... ... ... | | | | 3·33 |

as a confining fluid made the apparatus very convenient to handle, and that, provided due care were taken with the levelling on taking readings of the gas volume, the results were no less consistent than when water was used (Table III).

Table III. *Experiments in which mercury was used as confining fluid. Apparatus* 3 *and* 4.

| Initial reading of air pipette cc. | Final reading of air pipette cc. | Vol. of air added or abstracted (room temp. and pressure) cc. | Difference of level in clove oil manometer cm. | Constant (cmm. gas per mm. difference of level) |
|---|---|---|---|---|
| ·664 | ·436 | +·228 | 6·77 | 3·37 |
| ·446 | ·667 | −·221 | 6·60 | 3·35 |
| ·670 | ·333 | +·337 | 10·15 | 3·32 |
| ·340 | ·680 | −·340 | 10·18 | 3·34 |
| ·682 | ·206 | +·476 | 14·20 | 3·35 |
| ·211 | ·686 | −·475 | 14·16 | 3·35 |
| Mean constant for addition of gas ... ... ... ... ... | | | | 3·35 |
| Mean constant for abstraction of gas ... ... ... ... | | | | 3·35 |

The conclusion from the results of these experiments is that under the ordinary conditions of use of the differential apparatus the evolution of a given volume of gas in one bottle produces exactly the same change in level on the clove oil manometer as occurs in the opposite direction when the same volume of gas is absorbed from the same bottle.

It is of interest to compare this experimental result with that to which the mathematical theory of the differential apparatus leads. This was worked out originally by Sir Robert Ball [1908]; Hill [1915] calculates the relation between the actual reading observed and the difference between the amounts of gas

*absorbed* in each bottle, while Münzer and Neumann [1917] apply a somewhat different method of treatment for the case in which *evolution* of gas takes place in one bottle. But the mathematical comparison by similar methods of the two separate cases of absorption and evolution of gas seems not to have been undertaken. To consider the question, let us assume that we have an apparatus which is of the usual form, and in which, for the sake of a general case, the volumes of the left and right bottles are slightly unequal, namely $L$ and $R$ respectively. Each of these volumes includes the volume of the capillary tube down to the surface of the clove oil when this is standing level in the manometer.

Let $A$ be the area of cross section of the manometer tube (supposed uniform), and $P$ be the atmospheric pressure in mm. of the manometer liquid. The temperature at which the experiment is performed is constant throughout.

In the case of evolution of gas let a volume $x$ of gas (measured at atmospheric pressure and room temperature) be liberated in the left hand bottle, and let the resulting difference of pressure shown by the manometer be $2\epsilon$ mm.

Then by applying Boyle's law it is seen that the pressure in the left hand bottle

$$= \frac{(L+x)P}{L+\epsilon A}$$

while that in the right hand bottle

$$= \frac{RP}{R-\epsilon A}.$$

The difference between these is equal to the difference of level shown by the manometer,

$$\therefore \frac{(L+x)P}{L+\epsilon A} - \frac{RP}{R-\epsilon A} = 2\epsilon \quad \text{.....(1).}$$

On simplification, this expression becomes

$$x = \epsilon \frac{\left[A\left(\frac{L}{R}+1\right)+2\left(\frac{L}{P}-\frac{\epsilon AL}{PR}+\frac{\epsilon A}{P}-\frac{\epsilon^2A^2}{PR}\right)\right]}{1-\frac{\epsilon A}{R}},$$

and, on approximating by the binomial theorem, neglecting all terms of the order $\frac{1}{PR}$ and $\frac{1}{R^2}$, which are obviously small,

$$x = 2\epsilon\left[\frac{L}{P}+\frac{A}{2}\left(\frac{L}{R}+1\right)+\frac{\epsilon A}{P}+\frac{\epsilon A^2}{2R}\left(\frac{L}{R}+1\right)\right] \quad \text{.....(2).}$$

In the case of absorption of gas let an equal volume $x$ of gas (measured at atmospheric pressure and room temperature) be absorbed from the left hand bottle, and let the resulting difference of pressure shown by the manometer be $2\alpha$ mm.

Then by applying Boyle's law, as before, it is seen that the pressure in the left hand bottle

$$= \frac{(L-x)P}{L-\alpha A},$$

while that in the right hand bottle

$$= \frac{RP}{R+\alpha A}.$$

The difference between these is equal to the difference of level shown by the manometer,

$$\therefore \frac{RP}{R+\alpha A} - \frac{(L-x)P}{L-\alpha A} = 2\alpha \quad \text{.....(3).}$$

On simplification, this expression becomes

$$x = \alpha \frac{\left[A + \frac{AL}{R} + 2\left(\frac{L}{P} - \frac{\alpha A}{P} + \frac{\alpha AL}{PR} - \frac{\alpha^2 A^2}{PR}\right)\right]}{1 + \frac{\alpha A}{R}},$$

and, on approximating by the binomial theorem, neglecting all terms of the order $\frac{1}{PR}$ and $\frac{1}{R^2}$, which are obviously small,

$$x = 2\alpha\left[\frac{L}{P} + \frac{A}{2}\left(\frac{L}{R}+1\right) - \frac{\alpha A}{P} - \frac{\alpha A^2}{2R}\left(\frac{L}{R}+1\right)\right] \quad \text{.....(4).}$$

From the equations (2) and (4) it is evident that, for a given value of $x$, $\alpha$ will be sensibly equal to $\epsilon$ provided that the terms

$$\left\{\frac{\epsilon A}{P} + \frac{\epsilon A^2}{2R}\left(\frac{L}{R}+1\right)\right\} \text{ and } \left\{\frac{\alpha A}{P} + \frac{\alpha A^2}{2R}\left(\frac{L}{R}+1\right)\right\}$$

are of sufficiently small magnitude to be neglected in comparison with

$$\frac{L}{P} + \frac{A}{2}\left(\frac{L}{R}+1\right).$$

That this condition is fulfilled in practice may most readily be seen by taking an actual example referring to an apparatus of the usual dimensions. For instance, in an extreme case let the volumes of the bottles $L$ and $R$ be 24 and 25 cc., *i.e.* 24,000 and 25,000 mm.$^3$ respectively. Let the area of cross section of the bore of the manometer tubing be 1 mm.$^2$ and the atmospheric pressure 760 mm. Hg or about 10,000 mm. of the manometer liquid. If so much gas has been liberated or absorbed as to produce a difference of level of 100 mm. then

$$\frac{L}{P} + \frac{A}{2}\left(\frac{L}{R}+1\right) = 3{\cdot}38,$$

while

$$\frac{\epsilon A}{P} + \frac{\epsilon A^2}{2R}\left(\frac{L}{R}+1\right)$$
$$= {\cdot}007 \text{ (approximately).}$$

The neglected terms thus contribute a smaller share to the value of the constant than can be detected experimentally. The conclusion that under practical conditions the constant of the apparatus is the same no matter whether absorption or evolution of gas is taking place is thus supported by the theoretical as well as by the experimental results. In conclusion it might be mentioned that when the small terms in question are omitted from equations (2) and (4) they both reduce to

$$x = \frac{2\alpha L}{P} + \alpha AL\left(\frac{1}{L} + \frac{1}{R}\right)$$

which is seen to be identical with the formula given by Hill [1915], when it is remembered that Hill expressed the volume of gas absorbed in the apparatus as a fraction of the volume of the bottle in which the absorption takes place.

As mentioned above, Münzer and Neumann's method of treatment of the problem differs somewhat from the present one inasmuch as these authors express their results in a form involving a term $p$ which expresses the amount by which the general pressure in the apparatus exceeds the initial atmospheric pressure when evolution of gas takes place. The value of $p$ is given by

$$p = \frac{PV}{V - \epsilon A} - P + \epsilon \quad \text{.....(5),}$$

while the constant of the differential apparatus deduced in terms of this factor for the case of evolution of gas is equal to

$$\left[\frac{V}{P} + A\left(1 + \frac{2p}{P}\right)\right] \quad \text{.....(6).}$$

In both these latter equations $V$ is the volume of each of the bottles which are assumed to be identical in size. In order to show that this method of treatment leads to a formula identical with that given above in equation (2), it is necessary to avoid the approximations introduced by Münzer and Neumann [1917, p. 330].

In this way it is found that the exact form of formula (6) is

$$\frac{\left[\frac{V}{P} + A\left(1 + \frac{2p}{P} + \frac{p^2 - \epsilon^2}{P^2}\right)\right]}{1 + \frac{p}{P} - \frac{\epsilon}{P}},$$

but from (5)

$$1 + \frac{p}{P} - \frac{\epsilon}{P} = \frac{V}{V - \epsilon A} = \frac{1}{1 - \frac{\epsilon A}{V}}$$

so that (7) becomes

$$\left[\frac{V}{P} - \frac{\epsilon A}{P} + A\left(1 - \frac{\epsilon A}{V}\right)\left(1 + \frac{2p}{P}\right) + A\left(1 - \frac{\epsilon A}{V}\right)\left(\frac{p^2 - \epsilon^2}{P^2}\right)\right]$$

and by substituting the value of $p$ from (5) and neglecting terms of the order $\frac{1}{PV}$ and $\frac{1}{V^2}$ we obtain

$$\left[\frac{V}{P} + A\left(1 + \frac{\epsilon}{P} + \frac{\epsilon A}{V}\right)\right]$$

which is identical with the value of the constant expressed in equation (2) when $L$ and $R$ are both made equal to $V$.

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# XXIV. ON THE PHYSIOLOGY OF THE SILKWORM.

By ALEXANDER PRINGLE JAMESON
and WILLIAM RINGROSE GELSTON ATKINS.

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The digestive enzymes of Lepidoptera and other invertebrates appear to warrant a closer study than has as yet been accorded to them, more especially on account of the prevailing alkalinity of the secretions. Those of the silkworm were investigated by Sawamura [1900–1902], a summary of whose work, together with that of earlier investigators, is given below. It seemed of interest however to examine some of the actions more fully with the aid of indicators, whereby the hydrogen ion concentration could be measured with very approximate accuracy according to the method as improved by Clark and Lubs [1917]. In this connection the recent publication with coloured plates by Clark [1920] proved specially useful.

At the outset it may be remarked that it seemed advisable to discard such terms as stomach, intestine, colon, etc. which have a definite physiological connotation and to rely upon purely morphological terms which have no misleading association with vertebrate physiology. Behind the mouth there is the fore-gut, a thin-walled short tube, with no digestive cells according to Sawamura. Then follows the mid-gut with an expanded anterior portion, "the stomach," and a narrower posterior region, "the intestine." Both these regions have cells which secrete digestive enzymes. Behind the mid-gut lies the hind-gut, a short tube with no digestive power. The Malpighian tubes enter at the junction between the mid-gut and the hind-gut.

According to Kellner [1887] the silkworm, *Bombyx mori*, digests albumin, fat and carbohydrates except cellulose. Jousset [1874] showed that in *Blatta orientalis* the middle intestine was acid. Krukenberg [1880] concluded that trypsin predominates over pepsin in Insecta and other Arthropoda. Biedermann [1898] demonstrated that in *Tenebrio molitor* (Coleoptera) the upper mid-gut is acid, and the lower alkaline. It digests albumin, fat, disaccharides and starch, but not cellulose, the albumin being split up to amino compounds.

Sawamura found that in alkaline solution, which is the natural reaction, the enzymes of the silkworm digest starch rapidly, also but more slowly fat, with liberation of free acid. Fibrin is slowly acted on and gives the biuret reaction after two days at 36°. Gelatin is liquefied and freshly precipitated

caseinogen is dissolved. He concludes that tryptic, lipatic and diastatic enzymes are present, but that neither pepsin nor pepsinogen exists in the cells. The "intestine," namely the rear portion of the mid-gut, has a tryptic enzyme, but the others are absent. He further examined *Caligula japonica* Moor (Lepidoptera) and found that it contained a tryptic enzyme which gave peptones like pepsin, but in an alkaline solution. It differs from trypsin however in giving no amino-acids or tryptophan, the action stopping with the production of peptones. Starch was digested more slowly than by the silkworm as after six days at 36° the reaction-mixture still contained erythrodextrin, though maltose was also present as shown by the osazone. The existence of a feebly acting maltase was demonstrated by the fact that fermentation of the products of digestion by means of *Saccharomyces apiculatus* Reess, gave a little carbon dioxide after a few days. Since this yeast is free from maltase it is obvious that the glucose must have been produced by the digestive juices of the silkworm.

The interest in these results lies in the fact that the medium is alkaline. Now though the amylase of the human mouth, ptyalin, acts in an almost neutral solution, yet the diastase of the stomach, of plants and of taka-diastase preparation all act in acid solutions. Maltase moreover also acts in a neutral or faintly acid medium and is rendered inactive by a trace of alkali [Davis, 1916]. Yet in the insects mentioned these reactions are all in progress in an alkaline medium and cease when the reaction is made acid as Sawamura has shown.

It appeared of interest therefore to test for some of these enzymes taking into account the precise reaction of the medium.

*Hydrogen ion concentration of* Bombyx mori.

| Secretion or tissue | $P_H$ | Notes |
|---|---|---|
| Blood of worm No. 1 ... ... | 6·8 | Almost colourless liquid from haemocoel. Indicator, bromo-thymol blue |
| Reservoir of silk duct ... ... | 6·6–6·4 | The tissue was yellowish, so 6·8 may be a more correct value |
| Food and gastric juice, fore part of mid-gut ... ... ... | 9·0 | Fed on mulberry leaves 5.30–9 a.m. Tested 10.15 a.m. Indicator, thymol blue |
| Ditto from hinder part of mid-gut ... ... ... ... | 9·2 | Ditto |
| Hind-gut faeces ... ... | 8·4 | Indicator, cresol red |
| Mulberry leaf ... ... ... | 5·0 | Darker old leaf. Indicator, methyl red |
| | 5·0–5·2 | Light green younger leaf |
| Blood of worm No. 2 ... ... | 6·6 | Indicators bromo-thymol blue and bromo-cresol purple |
| Silk duct ... ... ... | 6·6–6·4 | — |
| Fore part of mid-gut, contents | 9·4 | No food given since 9 a.m. Killed 2 p.m. |
| Fore part of mid-gut, contents | 9·4 | From worm No. 3, badly diseased with pebrine, fed as the healthy worm No. 2 |
| Worm No. 4, healthy ... ... | 8·6 | Whole worm mashed up. Indicator, cresol red |
| Worm No. 5, highly pebrinised | 8·6 | Tint exactly matched that of No. 4 |
| Moth, hind-gut ... ... | 5·2 | Full of waste products. Indicator, methyl red |
| Moth, fore part mid-gut ... | 5·8 | Ditto, but indicator bromo-cresol purple |

From the foregoing it may be seen that the blood is almost neutral, having only a very faint acidity. The silk duct and its reservoir, which project into the haemocoel, have almost the same reaction, the slightly higher acidity values recorded being due in part to error introduced by the natural yellow of this tissue. It should be remarked that the reactions were judged by mixing indicator and tissue or secretion on white porcelain and matching against Clark's coloured plates and drops of standard solutions of known $P_H$ value. The very definite strong alkalinity of the digestive secretions is clearly shown in both the normal and highly diseased worms; no alteration in this reaction could be found in the latter. The strong alkalinity decreases somewhat towards the posterior end of the alimentary canal. It is noteworthy that after the metamorphosis the reaction of the gut has changed from alkaline to a fair degree of acidity. This is apparently due to the loading of the gut with waste products, for the moth does not feed.

It was thought possible that the alkalinity of the digestive juice of the worm might be due to the slow liberation of ammonia by bacterial action. This was disproved however by the fact that in a worm starved for a day and fed for twenty minutes before being killed the fore part of the mid-gut was found full of liquid with an alkalinity $P_H = 9{\cdot}6$–$9{\cdot}8$. The hind part, with but little food in it, gave $P_H = 9{\cdot}0$. Both portions of the mid-gut gave $P_H = 9{\cdot}6$–$9{\cdot}8$ in a similar worm killed fasting.

There is thus no doubt that the acid mulberry leaf is rapidly neutralised by a highly alkaline digestive juice and that the breaking down of starch and disaccharides must be accomplished in an alkaline medium, though the best known amylases are inactive under these conditions.

*Enzymes of the silkworm.*

The rapidity with which the digestive juice acts on a 0·4 % (approx.) solution of potato starch was shown by the following: 2 cc. of the starch solution diluted to 10 cc. mixed with three drops of the juice at 20°.

| Time in minutes | Colour with iodine solution | Notes |
|---|---|---|
| 0 | Blue | $P_H$=9·6 for the diluted |
| 2 | Blue | juice, no change in $P_H$ |
| 5 | Purple | effected by dilution |
| 6 | Brownish red | |
| 7 | Faint reddish | |
| 9 | Faint colour | |
| 10 | No colour | |

There is thus an active diastase in the secretion. The resulting solution reduced Fehling's solution.

Sawamura concluded that a feeble maltase was present in the secretion; our results are in agreement with this as after three drops of secretion had stood with 10 cc. of 0·16 % maltose for 24 hours at about 20° in presence of toluene it was found that of the control solution 48·4 cc. were required to reduce 10 cc. of Fehling's solution, whereas the solution with the secretion

required only 44·7 cc. A feeble invertase action could be detected in the digestive secretion after 20 hours.

On testing this secretion with benzidine and with α-naphthol no evidence of oxidase action could be found, nor was any shown on the addition of hydrogen peroxide, though a feeble catalase action was shown.

On testing the faintly yellowish liquid of the haemocoel, the "blood," no digestive action on starch could be observed, but the presence of invertase was demonstrated, also of a maltase of much the same degree of feeble activity as that shown by the digestive secretion of the mid-gut. No oxidase action was observed, even with the addition of hydrogen peroxide, but catalase action was found.

The carbohydrate metabolism of the silkworm appears therefore to begin with the digestion of starch and the further splitting up of part of the maltose formed in the mid-gut. The remainder of the cane sugar and maltose appears to be absorbed by the haemocoelic liquid and to be further broken up by it as required for metabolism. The "blood" shows no evidence of having a respiratory function as oxidases are absent. Respiration is provided for by the numerous tracheae found throughout the body of the worm.

## Summary.

1. The blood of the silkworm, *Bombyx mori*, has a hydrogen ion concentration $P_H = 6·8$ and that of the silk gland is very similar. The digestive secretion is strongly alkaline, for this $P_H = 9·0–9·8$. The reaction of the hind-gut is less strongly alkaline, $P_H = 8·4$.

2. The gut of the moth is acid, $P_H = 5·2–5·8$.

3. No difference could be detected between the $P_H$ values of the digestive secretions of normal and highly pebrinised worms, nor between their general body reaction.

4. A peculiar diastase is present in the digestive juice of the silkworm, which is active in strongly alkaline solution. A maltase is also present.

5. A feeble maltase and a more active invertase are present in the blood or haemocoelic fluid. Oxidases are absent from both blood and digestive juice, but catalase action is shown by both.

In conclusion we wish to acknowledge our indebtedness to the Director of the Agricultural Research Institute, Pusa, Behar, India, for facilities in carrying out this research.

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# XXV. THE INFLUENCE OF REACTION ON COLOUR CHANGES IN TRYPTOPHAN SOLUTIONS.

By ELFRIDA CONSTANCE VICTORIA MATTICK, and ROBERT STENHOUSE WILLIAMS.

(*From the National Institute for Research in Dairying, University College, Reading.*)

(*Received February 18th, 1921.*)

The work which is outlined below is a continuation of that already described [Cornish and Williams, 1917; Venn, 1920].

In the first paper a study was made of the colour changes produced by a number of strains of two groups of organisms, gram-negative alkaline and *B. proteus vulgaris*, upon caseinogen and upon some of its degradation products. In the course of this work it was noticed that the gram-negative alkaline organisms produced very marked colours on tyrosine, and only slight colours on the other amino-acids used, while the strains of *B. proteus vulgaris* produced definite colours on tryptophan and only slight colours, or none at all, on the others.

For these reasons the amino-acid tyrosine and one strain of the group of bacilli called gram-negative alkaline were chosen for an investigation into the influence of the reaction upon the colour production of this group of organisms, and the results of these experiments were given in the second paper previously mentioned.

The subject of the present paper is the influence of the reaction upon colour production in tryptophan solutions by *B. proteus vulgaris*. One strain ($C_{20}2B_2$) of this organism which was used in the original work was chosen, and after replating, was tested upon sugars, etc. with results which corresponded with those previously described [Cornish and Williams, 1917]. The technique of the $P_H$ determinations was similar to that adopted for the tyrosine solutions [Venn, 1920].

The tryptophan was prepared by Hopkins and Cole's method and a solution of 0·05 % was made and steamed for half an hour on each of three successive days. It was then filled out into Erlenmeyer flasks in quantities of 90 cc. and caustic soda (concentration before sterilisation 0·004556 g./cc.) was added in varying quantities to eleven, and hydrochloric acid (concentration before sterilisation 0·02421 g./cc.) to two of the solutions in these flasks. At a later date hydrochloric acid of 1/10 of this concentration was added to four

others in an endeavour to obtain $P_H$ values between 8·3 and 2·5 but the table shows that this attempt was unsuccessful. After the addition of caustic soda or hydrochloric acid the experimental solutions were kept for about two and a half months before the inoculations were made. All the work was carried out under sterile conditions.

Before this series of solutions was made preliminary experiments had been carried out over a range corresponding approximately to reactions of − 80 to + 20 Eyre's standard and colours had been produced from − 30 to + 2. The tryptophan itself was found to be approximately + 2·5 (Eyre's standard). The quantities and concentrations of the caustic soda and hydrochloric acid used in the later experiments were calculated to give reactions within which this colour producing range would be included.

At the time of the inoculation each solution was tested for sterility and no evidence of contamination was ever found. Each solution was then divided into three parts; the $P_H$ of one was determined, the second was inoculated with *B. proteus vulgaris*, and the third was kept as a control. The control and the inoculated solutions were incubated at 30° for seven days, when they were removed from the incubator and kept at laboratory temperature for another seven days, at the end of which time the $P_H$ of both portions was determined. Tests for growth in the inoculated solutions and for sterility of controls were made. Again, no evidence of contamination in the controls was found.

Frequent examinations for colour production were made by comparison with the controls.

The table shows that the organisms did not grow or produce colour when inoculated into tryptophan alone (solution 6), and a similar negative result was obtained with acidities varying from $P_H$ 1·8 to 2·5 (solutions 1 to 5 to which hydrochloric acid had been added). In those solutions to which caustic soda had been added, growth was found, accompanied by colour production, in solutions nos. 7 to 13 inclusive; growth was demonstrated but no colour was produced in solutions nos. 14, 15 and 16, while solution no. 17 was found to be sterile and no colour was produced. This shows that the range of reaction within which colour production was found in this series was between $P_H$ 8·9 and $P_H$ 9·4. The colours produced in the different solutions showed a definite sequence from deep orange in solution no. 7, passing through orange and deep yellow, to a light yellow until it disappeared at no. 14. These results confirmed those already obtained in the preliminary series in which colours were found over a range of − 30 to + 2 but were entirely absent at − 40 and + 4·5 (Eyre's standard).

The table given below is a summary of the results obtained.

| Original solutions. Each contains 90 cc. of 0·05 % tryptophan solution. Varying amounts of NaOH (1 cc. = 0·004556 g.) or HCl (1 cc. = 0·02421 gr.) or HCl (1 cc. = 0·002421 gr.) | | | | Inoculated solutions | | |
|---|---|---|---|---|---|---|
| No. of solution | No. of cc. NaOH or HCl | $P_H$ at time of Inoculation | Control solutions $P_H$ | Growth | Colour | $P_H$ |
| | 1 cc. = 0·02421 g. HCl | | | | | |
| 1 | 2·05 | 1·73 | 1·73 | – | — | 1·68 |
| 2 | 1·3 | 2·12 | 1·86 | – | — | 1·86 |
| | 1 cc. = 0·002421 g. HCl | | | | | |
| 3 | 10 | 2·10 | 2·16 | – | — | 2·18 |
| 4 | 5 | 2·32 | 2·24 | – | — | 2·23 |
| 5 | 3 | 2·36 | 2·53 | – | — | 2·55 |
| 6 | Tryptophan only | 8·24 | 8·12 | – | — | 8·19 |
| | 1 cc. = 0·004556 g. NaOH | | | | | |
| 7 | 0·75 | 8·95 | 8·63 | + | Deep orange | 8·95 |
| 8 | 1·25 | 8·89 | 9·03 | + | Deep orange | 8·95 |
| 9 | 2·25 | 9·19 | 9·07 | + | Orange | 9·07 |
| 10 | 3·25 | 9·27 | 9·21 | + | Deep yellow | 8·98 |
| 11 | 4·25 | 9·32 | 9·27 | + | Greenish yellow | 9·35 |
| 12 | 4·85 | 9·36 | 9·37 | + | Yellow | 9·36 |
| 13 | 5·6 | 9·44 | 9·44 | + | Light yellow | 9·41 |
| 14 | 7·0 | 9·47 | 9·51 | + | — | 9·50 |
| 15 | 8·0 | 9·51 | 9·50 | + | — | 9·46 |
| 16 | 9·0 | 9·47 | 9·60 | + | — | 9·62 |
| 17 | 11·0 | 9·58 | 9·57 | – | — | 9·57 |

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# XXVI. METHODS OF MEASURING THE OPACITY OF LIQUIDS.

By J. HOLKER.

*From the Department of Pathology, University, Manchester.*

(*Received February 18th, 1921.*)

## Part I.

The writer has long been of the opinion that, given a suitable and sufficiently accurate method of measuring the opacity of a liquid, it would be possible not only to study more closely than hitherto the mechanism of serum reactions, but also to evolve methods of importance in practical diagnostic and therapeutic pathology.

As none of the several available methods of measuring turbidity was suitable for the investigation proposed, it was necessary to devise one. In the first form of apparatus, which was worked out in 1918, it was decided to use the loop commonly employed in bacteriological routine for making subcultures. The loop itself was bent at the end of the wire at a right angle and the shaft was fixed to a sliding micrometer provided with a scale and vernier. In this way the depth of the loop below the surface of the emulsion at which it just disappeared from sight could readily be determined. This method was, however, discarded as not being sufficiently accurate. Recently Gates [1920] has published exactly the same principle for standardising vaccines. Priority for this particular method must, however, be given to Don and Chisholm [1911] who used it in connection with water analysis.

In the second form of apparatus which is entirely new in principle and which is the one described at length in this article, the wire was fixed in a constant position, whilst the depth of the liquid under examination was made to vary. This opacimeter (see Fig. 1) consisted of a tube of resistance glass rather more than 250 mm. long and 20 mm. wide, along the side of which was etched a millimetre scale. The bottom of the tube was blown flat; at the side, about 20 mm. from the bottom, was a narrow tube provided with a small tap, so that liquid could be run off to any depth. Running across the flat bottom of the tube on the outside from left to right was a piece of wire of known diameter, determined by means of a screw micrometer, and supported by two small blobs of plasticine about 1·5 mm. in diameter. The wire used was one of the fine copper shreds which can be obtained from the flexible material used in connecting up a wall plug with a portable electric lamp. It was blackened by carefully passing it through the Bunsen flame. The opacimeter was supported by a simple metal stand round the bottom of the tube, care being taken that, when the apparatus was standing upright, the wire and

the plasticine were clear of the bench. When a sufficiently turbid liquid was placed in the tube, the wire could not be seen. But on turning the tap the liquid could be run off until the wire just appeared. The reading was facilitated by the presence of the blob of plasticine at each end of the wire. These blobs being relatively much larger than the wire became visible first, and therefore acted as signals for a careful approach to the end-point. The end-point having been determined, the depth of the liquid was read off on the millimetre scale etched on the outside of the tube, the bottom of the rather flat meniscus being

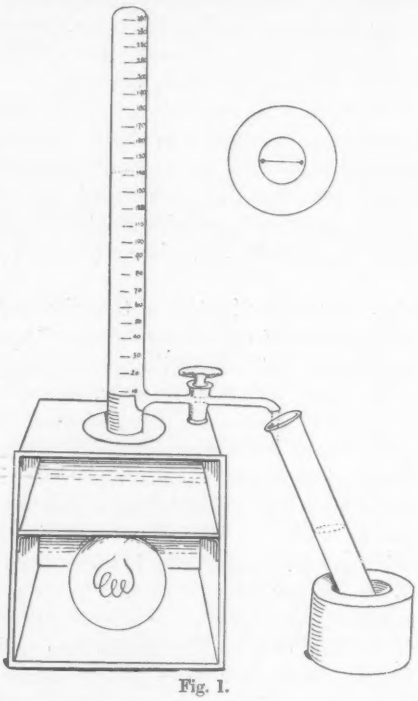

Fig. 1.

taken as the correct level. The reciprocal of this reading was taken as a measure of the opacity of the liquid. As the reading of the scale of the opacimeter varied from 20 to 250, the turbidity varied from 1/20 to 1/250, *i.e.* from $500 \times 10^{-4}$ to $40 \times 10^{-4}$. In writing up results the factor $10^{-4}$ was always understood.

To provide for a suitable source of light, a sheet iron box was made 5 inches high, 5 inches from left to right and 6 inches from front to back. In the lower part of the back of the box was a hole to support an electric bulb. At a distance of 3 inches from the bottom of the box and just above the upper level of the bulb were two shelves, one on each side, to support a sheet of opalescent glass

2 mm. thick, which served to diffuse the light from the incandescent filaments of the lamp. In this way a beautiful light was obtained of slightly yellowish hue. The roof of the box was formed by a loose plate containing a hole somewhat wider than the opacimeter tube and directly over the brightest part of the opalescent screen. Over the hole the opacimeter was made to stand, so that the wire and plasticine blobs appeared very clear and black against the contrasting background. The front of the box was provided with a sliding door. In order to cut off all light other than that coming from the opalescent screen, a sheet-iron tube 35 mm. wide and painted with lamp-black was dropped over the opacimeter during the time a reading was made. Between the lamp and the wall plug were placed an accurate ammeter and a resistance, so that the current could be readily regulated to within 0·02 amp.

The principle of the method depends primarily on the fact that, under the conditions of the apparatus, only particles above a certain size scatter the light. Ions or molecules in solution are so small compared to the average wave length of the light used that their scattering power may be regarded as nil. Particles of the size of amicrons probably scatter the light to a very slight extent, submicrons more markedly and microns powerfully. Large particles cast a shadow.

The light entering through the bottom of the tube casts a shadow above the wire, so that it is convenient to speak of a "shadow" space and a "bright" space. In the bright space the intensity of the light diminishes with increasing depth probably by the logarithmic law $y = ke^{-ax}$, where $y$ is the intensity of the light at the depth $x$ of the suspension, $k$ is a constant which measures the intensity of the incident light, $a$ is a constant which depends on the number, size and shape of the particles scattering light, and $e$ is the base of the natural logarithms. On the other hand, owing to the scattering of light from the bright into the shadow space, the intensity of light in the latter increases probably logarithmically with the depth up to a certain level. But any light thus transferred to the shadow space is subject to the same law as that in the bright space, so that there must come a level higher up at which the intensity of light in the shadow space approaches zero again. In other words the intensity of light in the shadow space passes through a maximum, and beyond the maximum it approximates more and more to the intensity in the bright space. On this hypothesis the curves representing the intensity of light at different depths are depicted in Fig. 2.

Since the determination of the end-point or depth of supposed equality involves a discrimination between two intensities of light, Weber's law applies, and there will be a minimum proportional difference of intensity below which it is impossible for the eye to distinguish the difference in the two spaces. Therefore if, at the end-point, $a$ is the intensity of light in the bright space, and $b$ is that in the shadow space, then $a/b$ may be equal to, but cannot be less than, the minimal proportional difference of intensity.

When the intensity of the source of light is increased, other conditions

remaining constant, the intensity in both the light and shadow space at the original depth of supposed equality, will be increased in the same proportion. For example, if the incident light is doubled the ratio will be $\frac{2a}{2b} = \frac{a}{b}$, so that the end-point remains the same for variations in the intensity of the incident light. That wide variations in the candle power of the electric lamp have no effect on the readings is shown by the following experiments. A suspension was taken of liquid paraffin in water. The strength of the suspension was

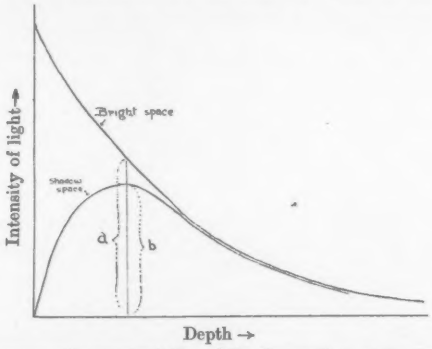


Fig. 2. Diagrammatic representation of the theory that, at the depth of supposed equality of light in the shadow and light spaces, there is a minimum proportional difference in the intensity of the light in the two spaces, that is, the ratio $a/b$ is a minimum. When the incident light is increased, the light in both spaces is increased in the same proportion, so that the ratio remains the same.

adjusted so that the readings were of a convenient height. The first reading was made with a 16 c.p. lamp as the source of light, and the resistance was regulated so that the light was a bright one. Under these conditions the reading was 126·2. Keeping to the same conditions but now using a 32 c.p. lamp, the reading was 125·7. Again using a suspension of washed red corpuscles in physiologically normal saline with a 16 c.p. lamp and a bright light the reading was 187·7, with a 32 c.p. lamp and a bright light the reading was 187·8. The very slight differences observed between the readings were well within the experimental error. The detailed results of the experiment are collected together in Table I.

Table I. *Showing that wide variations in the candle power of the electric lamp used as the source of light, produce only negligible changes in the readings of the opacimeter. The dull light was produced by currents of* 0·26 *and* 0·42 *amp. for the* 16 *and* 32 *c.p. lamps respectively. The bright light was produced by* 0·30 *and* 0·59 *amp. respectively.*

| Luminosity of lamp as regulated by resistance coil | Depth of suspension at end-point | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Liquid paraffin suspension | | | | Red corpuscle suspension | | | |
| | A | | B | | C | | D | |
| | 16 c.p. lamp | 32 c.p. lamp | 16 c.p. lamp | 32 c.p. lamp | 16 c.p. lamp | 32 c.p. lamp | 16 c.p. lamp | 32 c.p. lamp |
| Dull ... | 119·7 | 120·7 | 210·7 | 212·0 | 101·7 | 102·2 | 176·8 | 177·2 |
| Bright ... | 126·2 | 125·7 | 222·7 | 222·2 | 106·7 | 105·7 | 187·7 | 187·8 |

The figures in Table I show however that it is advisable to keep to one lamp and to regulate it by means of the resistance so that the amperage is constant, because the colour, and therefore the luminosity of the lamp, varies with the heat of the filaments. Thus with a 16 c.p. lamp, which with an amperage of 0·26 gave a dull slightly reddish light, the reading of a suspension of red corpuscles was 101·7; with an amperage of 0·28 giving a moderately bright yellowish light, the reading was 105·2, with an amperage of 0·30 giving a very bright slightly yellowish light, the reading was 106·7. Similar results were obtained with a suspension of liquid paraffin.

Occasionally it was necessary to use different opacimeters during one and the same experiment. A preliminary test was therefore made to determine how far the readings varied with different opacimeters, the same suspension being used in each case. With six opacimeters made according to the pattern described, the readings of a sample of milk diluted one thousand times with distilled water were as follows: 127·0, 126·3, 126·4, 127·0, 126·2, 126·6. The mean of these was 126·6. The respective differences from the mean were + 0·4, − 0·3, − 0·2, + 0·4, − 0·4, 0·0, and the mean difference from the mean was 0·3 or a percentage of 0·2. Hence it was permissible to use any opacimeter made according to standard.

Again, it was necessary from time to time for more than one observer to take readings during an experiment. Of the following observers *A* had good visual acuity, *B* moderately good, and *C* poor, though corrected to some extent by glasses. Using the same opacimeter the following results were obtained:

| Suspension | Mean of all observers' readings | Percentage difference from the mean of each observer's readings | | |
|---|---|---|---|---|
| | | % *A* | % *B* | % *C* |
| No. 1 | 52·7 | +0·6 | +0·4 | −0·8 |
| 2 | 109·9 | +0·6 | +0·1 | −0·6 |
| 3 | 185·8 | +0·1 | +0·6 | −0·7 |
| Average percentage difference from the mean | | +0·4 | +0·4 | −0·7 |

It is to be pointed out that the observer *C* had not only weak eyesight but was unfamiliar with the apparatus at the time the above readings were made.

If two or more observers frequently work together a factor for correction of their readings is easily calculated. Suppose the average percentage differences from the mean are $+ x, - y, + z$ respectively

$$\frac{\text{Corrected reading}}{A\text{'s observed reading}} = \frac{100}{100 + x}$$

$$\frac{\text{Corrected reading}}{B\text{'s observed reading}} = \frac{100}{100 - y}$$

and so on. Therefore the respective factors for correction are

$$\frac{100}{100+x}, \quad \frac{100}{100-y}, \quad \frac{100}{100+z}.$$

For example the correction factors for Preston (very good eyesight) and Ponting (good eyesight) who frequently worked together were 0·994 and 1·006 respectively.

That one observer could reproduce readings for the same suspensions with a high degree of accuracy was repeatedly observed. For example observer *A* obtained the following consecutive readings with a suspension of *B. coli* in normal saline: 105·0, 104·8, 105·0, 104·5, 104·5, 104·3, 104·2, 104·0, 104·0, 104·0. The mean of these readings was 104·4, and the highest, lowest and mean differences from the mean were 0·6, 0·1, 0·3 respectively. The measure of the accuracy was therefore 0·29 %.

For accurate work it is preferable to work with suspensions having a reading of not much less than 100, because at or above this level a difference of, say, one millimetre in consecutive readings produces only a slight degree of error. Usually the average of three readings was taken, but for very accurate work more readings were taken. The highest degree of accuracy is obtained by working in a dark room, but for practical work this was not found necessary. It has been found that beginners tend to run off too much and obtain too low a reading. To avoid this it is advisable to make a preliminary observation by running off quickly until the plasticine blobs appear, and then running off more slowly until the wire just appears. After refilling the apparatus, the height of the liquid should be adjusted at about 10 to 15 mm. above the preliminary reading, and the tap then very carefully turned on so that the delivery is in slow drops. One drop a second was satisfactory, and at this speed three drops corresponded closely to a fall of 1 mm. This method of making a determination of the end-point also avoided eye fatigue, as the average duration of a reading was only about 25 seconds. The end-point having been determined, the reciprocal was found either by the use of a slide rule or more conveniently from a table of reciprocals such as is found in Castle's tables [1917].

Occasionally the opacity of a liquid was so slight that the reading was greater than 250 mm. (the height of the tube). When this was the case the method described below was used. The stands of two opacimeters were cut so that the tubes could lie close together. The circular cover was replaced by a rectangular one passing over both tubes but having a partition down the middle to provide a separate compartment for each. The loose roof of the box with a circular hole was also replaced by one having a rectangular hole about the dimension of the bases of the two tubes. Into one tube was placed the liquid of unknown opacity, the tube being nearly full. Into the other tube was placed an emulsion of similar colouring to the unknown and having a reading somewhat less than 250 mm. As a general standard, a stable suspension was obtained by diluting a few drops of Angier's emulsion of paraffin with distilled water. Such a suspension with a reading of 200 had repeatedly been found unchanged at the end of the day. The two tubes having been filled with the respective liquids, the emulsion of known opacity was then run off until its opacity matched that of the unknown.

Then if $x$ = required reading of unknown emulsion,

$p$ = depth of unknown emulsion actually present in tube,

$q$ = reading of known emulsion, the reciprocal of which is the measure of its opacity,

$r$ = reading of known emulsion which matches the opacity of the unknown,

$$\frac{x}{p} = \frac{q}{r}, \quad \therefore x = \frac{pq}{r}.$$

The reciprocal of $x$ then gave the opacity of the unknown. This formula was tested by preparing known dilutions of emulsions whose readings came below 250 in the original. By this means a turbidity as low as 8·7 has been measured.

As in experimental work it was often a question of measuring the number of particles in a suspension, it became a matter of importance to test whether the ratio between the opacity and the concentration was constant, that is if $T/C = k$. As the scattering power of the suspension depends on the number, size and shape of the particles, one would expect that, provided the size and shape remained constant, the turbidity would vary directly as the number of particles. This was found to be the case. For example, a suspension of *gonococci* in physiologically normal saline gave the following results, 100 being taken as the concentration of the strongest suspension.

| Concentration $C$ | Opacity $T$ | $T/C$ |
|---|---|---|
| 100 | 102·0 | 1·020 |
| 66·6 | 67·6 | 1·015 |
| 50·0 | 51·0 | 1·020 |
| 33·3 | 33·8 | 1·015 |

Similarly pure cultures of *Staphylococcus aureus*, *B. coli* and suspensions of washed red corpuscles obeyed the same law, so that once the concentration and opacity had been correlated, it was sufficient afterwards to measure the opacity in order to determine the concentration.

## Part II.

The apparatus described in Part I of this paper proved very useful in measuring the opacity of suspensions of which the amount was not very limited. In serum reactions however the amount of material is usually strictly limited and as the author was anxious to study these reactions he was obliged to modify the apparatus.

In the new form the lighting arrangement is similar to that used in the first method, but the liquid whose opacity is to be determined is placed in a narrow tube 50 mm. long, and holding about $1\frac{1}{4}$ cc. As will be seen from Fig. 3 this micro-opacimeter is placed between the source of light and a macro-opacimeter containing a standard suspension. Before measuring the unknown suspension the reading of the standard is taken with the micro-tube containing distilled water only. Then a micro-opacimeter containing the

unknown suspension is substituted for the one containing the distilled water and the reading of the standard again taken. The second reading is lower than the first, and the lowering is in proportion to the turbidity of the unknown.

Let $S$ = reading of standard when distilled water is interposed,

$M$ = difference of readings of standard when distilled water and unknown respectively are interposed,

$X$ = required reading of unknown.

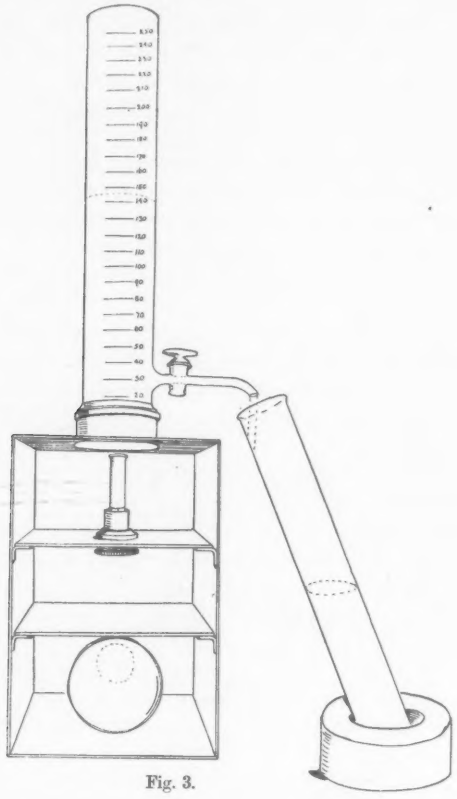

Fig. 3.

Then $X/50 = S/M$, $\quad \therefore X = 50S/M$;

$$\therefore \text{ turbidity} = \frac{1}{X} = M/50S.$$

In practice the standard was always adjusted at the beginning of the day to a reading of 200, so that the turbidity formula became $\frac{M}{50 \times 200} = M \times 10^{-4}$. As the factor $10^{-4}$ is always understood in turbidity determinations, the factor $M$, which represents the difference between 200 and the reading of the standard

with the unknown interposed, gives us at once without any calculation the opacity of the unknown.

The standard used was a stable one made by diluting a small drop of Angier's emulsion of liquid paraffin with distilled water. Such a suspension keeps constant throughout the day, and is found to have changed only slightly even on the following day. It was prepared fresh each morning.

The micro-opacimeter tube is made of Jena glass about 5·5 mm. wide. Pressed firmly against the ground edge of the bottom of the tube is a plate of optically clear glass. In this way any possible error from the use of a cement joint is avoided. The joint was found to be completely water-tight. Around the lower part of the tube is cemented a brass collar provided with a thread. Screwed firmly on to the collar is a brass cap which is pierced by a hole of definite diameter so as to allow a definite amount of light to pass up the tube. Inside the cap is a rubber ring between which and the tube lies the plate of glass mentioned. The upper edge of the tube is ground flat, and when the opacimeter has been slightly overfilled so as to produce a convex meniscus a loose optically clear glass disc is dropped on to the top of the tube. In this way the upper surface of the liquid in the tube is rendered quite flat. With the narrow tubes used it was found that the sharply curved meniscus, which is formed at the upper surface of the liquid, acted as a powerful lens, and considerably altered the apparent diameter of the shadow. Though the length of the micro-tubes was adjusted by grinding to as near 50 mm. as possible, it was finally determined with accuracy by means of a travelling microscope, and a factor for correction calculated for each tube.

The macro-opacimeter is also provided with a metal collar and cap holding a glass disc against the bottom of the tube. The macro-tube is rather longer than 250 mm. and 38 mm. wide. It was found advantageous to increase the width of the tube beyond that used in the first form of apparatus, as by so doing the meniscus becomes sufficiently flat to be negligible in its action as a lens in altering the diameter of the shadow. During a reading a blackened metal tube was dropped over the large tube in order to cut off side light. Similarly to cut off top light the metal tube was provided with a metal cap containing in the middle a short tube 45 mm. long, against the aperture of which the eye could be placed. During the reading the front of the lamp box was closed by means of a sliding door.

A further improvement on the method described in Part I was that instead of a separate wire being placed at the bottom of each tube, only one wire was used. This was placed between two optically clear discs of glass which were pressed together by two brass rims having apertures 10 mm. wide. In order that the wire might be steady and retain its position between the glass plates, two small blobs of plasticine were also pressed flat near the ends. These blobs of plasticine also acted as signals for the approach of the end-point. The metal cell containing the wire was supported in a shelf in a position just above the highest part of the opalescent screen and directly in line with the light passing

up the two tubes. A better method of producing the shadow would be to replace the wire by a "graticule" or black line of known diameter etched by a photographic process on to the glass disc.

## Part III.

The apparatus described in Part II has proved exceedingly useful for studying the interaction of electrolytes, suspensoids and emulsoids, and especially for covering a wide ground with only a limited supply of material. It was felt however that a more perfect apparatus would be one in which the reading could easily be taken from either direction, that is, not only when the shadow just appeared as in methods I and II, but also when the shadow just disappears. Preliminary efforts were made with the apparatus described in Part II by connecting a gas analysis funnel by means of rubber tubing to the side tube of the macro-opacimeter. By raising or lowering the funnel the level of the standard could be moved in either direction at will. However, it was thought that a better method would be to use a plunger which could readily be moved up and down with a rack and pinion. The plunger consists of a hollow tube of brass on the bottom of which is pressed an optically clear plate of glass in the manner described in Part II. The height of the under surface of this disc from the bottom of the macro-opacimeter is registered in a millimetre scale on the side of the plunger. As a further refinement a fine adjustment has been provided so that the depth of the plunger can be determined with an accuracy of one tenth of a millimetre. The initial readings are first made by means of the coarse adjustment, so as to get a fairly accurate idea of the position of the end-point. The final readings can then be made with the fine adjustment. The fine adjustment is intended primarily for taking the readings of almost transparent liquids, and is especially useful therefore when the readings are from 0 to 20.

Still further refinements which the author hopes to try are the reduction of the width of the shadow and the use of a telemicroscope in the hollow plunger. The apparatus described in Part II proved exceedingly sensitive, but the refinements now suggested should render it still more so.

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# XXVII. THE RELATION BETWEEN THE NUMBER AND SIZE OF RED CORPUSCLES AND THE OPACITY OF THEIR SUSPENSIONS.

By J. HOLKER.

*From the Department of Pathology, University, Manchester.*

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In a recent investigation on the standardisation of vaccines [1921], the author was led to observe that a constant could be obtained for each kind of organism, which was not only useful in determining the number of bacteria per cubic centimetre of a suspension, but whose reciprocal was also a measure of the average size of the organism. It seemed advisable therefore to study more fully the influence of number and size of particles on the opacity of their suspension. Bacteria however did not lend themselves to exact methods owing to their small and varying size and shape. Mammalian red corpuscles, on the other hand, being much larger and of much more constant shape lent themselves very well to the proposed work, especially as reliable data had already been published as to their size and shape. For the purpose of this paper then, the red corpuscles of the animals quoted below were used.

| Animal | Diameter of red corpuscles | Authority |
|---|---|---|
| Sheep ... | 5·0$\mu$ | Vierordt [1888] |
| Rabbit ... | 6·9$\mu$ | Vierordt [1888] |
| Guinea-pig ... | 7·2$\mu$ | Gulliver [1875] |
| Man ... ... | 7·5$\mu$ | — |

As the medium in which the corpuscles were suspended was a solution of sodium chloride, it was first sought to determine the effect of varying concentration of saline on the opacity of a suspension of red corpuscles of constant concentration. The red corpuscles used were those of the sheep and were "washed" by the method commonly adopted in preparing them for the Wassermann reaction. This was as follows. Freshly collected sheep's blood having been defibrinated by shaking with glass beads, the red corpuscles were then powerfully centrifuged for 10 minutes. The supernatant defibrinated plasma was then decanted, replaced by 0·85 % sodium chloride, and the resulting suspension again centrifuged for 10 minutes. Decantation and renewal of saline, followed by a third centrifuging lasting half an hour, completed the process. When a suspension of corpuscles was required for experimental purposes the supernatant saline was decanted from the centrifuge tube and replaced by physiologically normal saline.

A suspension of washed sheep's corpuscles having been prepared and diluted with normal saline until the opacimeter reading was a fairly low one of about 50, the correct reading of this was then accurately determined by means of the apparatus recently described by the author [1921, Part I]. With this stock suspension the effect of endosmosis and exosmosis was measured. For testing the effect of endosmosis the stock suspension was diluted in a volumetric flask with varying amounts of hypotonic saline, and the opacity of each dilution determined and expressed as a percentage of the opacity of the stock suspension.

The results are collected together in Table I.

Table I. *Effect of endosmosis on the opacity of a suspension of red corpuscles.*

| Concentration of NaCl % | Opacity of suspension expressed as a percentage of the stock suspension |
|---|---|
| 0·85 | 100 |
| 0·799 | 89·3 |
| 0·765 | 81·6 |
| 0·731 | 64·1 |

The effect of exosmosis was determined by adding varying amounts of hypertonic saline to the stock suspension, a volumetric flask being used as before. The opacities of the suspensions having been determined, the results were again expressed as a percentage of the opacity of the stock solution. The results are shown in Table II and the curve is shown in Fig. 1.

Table II. *Effect of exosmosis on the opacity of a suspension of red corpuscles.*

| Concentration of NaCl % | Opacity of suspension expressed as a percentage of the stock suspension |
|---|---|
| 0·85 | 100 |
| 0·956 | 99·4 |
| 1·488 | 95·5 |
| 2·550 | 87·2 |
| 3·613 | 79·3 |
| 4·675 | 71·2 |

On plotting the opacity of the suspension against the concentration of sodium chloride, the curves are seen to be straight lines but sloping in opposite directions and at different angles from a point which represents the isotonic strength of sodium chloride. The two lines represent separate and distinct processes, which may be explained as follows. As a result of endosmosis, some of the red corpuscles are broken up, with a corresponding reduction in opacity. The reduction in opacity is proportional to the reduction in strength of the sodium chloride. In the case of exosmosis the corpuscles shrink, and the shrinkage is proportional to the excess of sodium chloride used. This steady diminution in size of the red corpuscles with increasing strength of the saline above the isotonic point produces a corresponding diminution of the

opacity. The curve representing the action of varying concentration of sodium chloride on the opacity of a given suspension of red corpuscles consists then of two parts, one limb showing the change in opacity due to the change in the number of corpuscles, the other limb showing the change in opacity due to change in size of the corpuscles, and the junction of the two lines represents very sharply the point at which there is no change either in the number or size of the corpuscles, *i.e.* the isotonic point. The effect of endosmosis is much more marked than that of exosmosis.

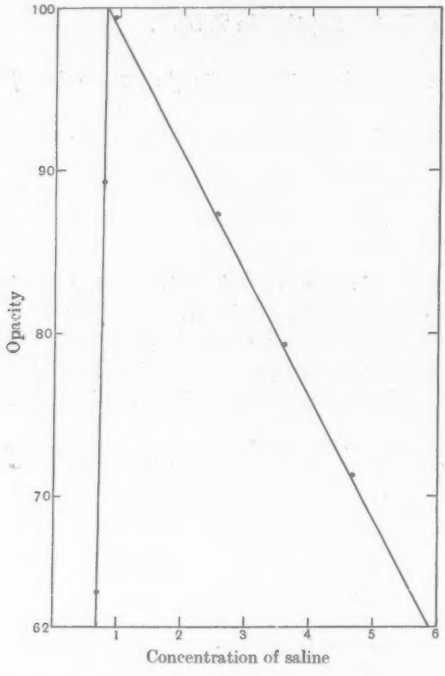


Fig. 1.

In the second series of experiments the concentration of the saline was maintained constant at 0·85 %, while the size of the corpuscle was made to vary by using the blood of the animals quoted above, "washed" corpuscles being used in each case. It was found that for each type of red cell the curve obtained on plotting the concentration of the red cells against the opacity of their suspension was a straight line, and the equation was of the simple order $T = kC$, where $T$ = opacity of the suspension, $C$ = number of red cells per cubic millimetre as determined by the haemocytometer, and $k$ = constant which represents the theoretical opacity produced by one red cell per cubic millimetre. To obtain as accurate a value of $k$ as possible, a suspension of red

cells was diluted a known number of times so as to produce a field in the haemocytometer showing about five or six corpuscles per square, and the whole field of four hundred squares then counted. At the same time the original suspension was diluted a known number of times so as to produce a convenient reading of about one hundred in the opacimeter, and the opacity accurately determined. The opacity and the cell count of the original suspension were then calculated and the ratio determined. In this way the following constants were obtained.

| Animal | Constant $k$ |
|---|---|
| Sheep ... | $4{\cdot}29 \times 10^{-6}$ |
| Rabbit ... | $8{\cdot}21 \times 10^{-6}$ |
| Guinea-pig ... | $8{\cdot}86 \times 10^{-6}$ |
| Man ... ... | $9{\cdot}50 \times 10^{-6}$ |

Once the constant $k$ has been determined for each animal, the number of red cells per cubic millimetre is readily and accurately determined from the opacity by the equation $C = \frac{T}{k}$. Evidently the constant $k$ is related to the size of the red corpuscles. So far as could be ascertained by watching with the microscope when the corpuscles fall under gravity in a specially made capillary tube whose walls were flat, the path taken was in general not vertical but sloping away from the vertical. The amount of light scattered by each corpuscle therefore may be taken as proportional to the incident light coming through the horizontal plane of projection of the area of an inclined circle whose diameter is equal to that of the cell, and therefore proportional to the area of the circle itself. The areas corresponding to the corpuscles examined were as follows:

| Animal | Proportional area of red corpuscles |
|---|---|
| Sheep ... | $19{\cdot}64\mu^2$ |
| Rabbit ... | $37{\cdot}39\mu^2$ |
| Guinea-pig ... | $40{\cdot}72\mu^2$ |
| Man ... ... | $44{\cdot}17\mu^2$ |

Bearing in mind that the constant $k$ represents the turbidity produced by one corpuscle per cubic millimetre, it was decided to determine the turbidity proportional to $1\mu^2$ (one millionth of a square millimetre), *i.e.* the following fraction was evaluated $\frac{k}{\pi r^2}$, where $r$ = radius of red corpuscles. The results were as follows:

| Animal | Opacity proportional to $1\mu^2$ of surface of red corpuscle per cm. of suspension $= k$/proportional area |
|---|---|
| Sheep ... ... | $0{\cdot}218 \times 10^{-6}$ |
| Rabbit ... | $0{\cdot}220 \times 10^{-6}$ |
| Guinea-pig ... | $0{\cdot}218 + 10^{-6}$ |
| Man ... ... | $0{\cdot}215 \times 10^{-6}$ |
| Average ... | $0{\cdot}218 \times 10^{-6} = a$ |

Obviously then the opacity produced by one millionth of a square millimetre of the surface of a red corpuscle per cubic millimetre of suspension was the

same for all the types of cell examined. This constant $\alpha$ appears to be a characteristic of the mammalian red corpuscle in general.

Since $$\frac{k}{\pi r^2} = \alpha,$$

$$\therefore r = \sqrt{\frac{k}{\pi a}}$$

$$= \sqrt{\frac{T}{\pi a C}}$$

$$= \sqrt{\frac{1}{\pi a C D}},$$

where $D$ = opacimeter reading.

$\therefore$ Diameter $$= 2\sqrt{\frac{1}{\pi a C D}}.$$

Evaluating $$2\sqrt{\frac{1}{\pi a}} = 2{\cdot}402 \times 10^3,$$

$\therefore$ Diameter $$= \frac{2{\cdot}402 \times 10^3}{\sqrt{CD}}.$$

To obtain the average diameter of a mammalian red corpuscle in suspension in physiologically normal saline it is sufficient to make a cell count and to determine the opacimeter reading of the suspension at the end-point.

Again, since $$\frac{k}{\pi r^2} = \alpha,$$

$$k = \pi a r^2$$

$$= \tfrac{1}{4}\pi a d^2,$$

where $d$ = diameter of corpuscle in $\mu$.

Evaluating $\frac{1}{4}\pi a = 0{\cdot}171 \times 10^{-6}$,

$$k = 0{\cdot}171 d^2 \times 10^{-6}.$$

Therefore the constant for other mammalian red corpuscles may readily be calculated from the published data for their diameters. Since the opacity produced by one millionth of a square millimetre ($1\,\mu^2$) of the surface of a red corpuscle per cubic millimetre of suspension is constant for the mammalian cell, it follows that the opacity of a suspension of red corpuscles is a measure of the total surface of such cells and, by inference, that opacity in general of a liquid is a measure of the total surface of the particles therein scattering light. Opacity does not measure the total surface of all matter present, because ions and molecules which are very small relative to the wave length of light, do not scatter light. Hence this peculiar property of opacity measurements should be useful in studying the course of reactions in which solute becomes amicrons, amicrons become submicrons, and submicrons become microns, or *vice versa*. Hence also in those reactions in physiology and pathology in which surface phenomena play a leading part, such as enzyme reactions and the Wassermann reaction, measurements of opacity should be of more value in elucidating the underlying phenomena than measurements of the quantity of material.

SUMMARY.

1. Endosmosis and exosmosis may be measured by means of the opacimeter.

2. By plotting the results of endosmosis and exosmosis the isotonic point may be accurately determined.

3. The opacity-concentration curve of a suspension of red corpuscles is a straight line with the equation $T = kC$, where $T$ = opacity, $C$ = concentration of corpuscles per cmm., and $k$ = constant, which varies with the animal, and is a measure of the size of the red corpuscles.

4. The number of red corpuscles in a suspension can be readily and accurately determined from the opacity.

5. The average size of mammalian red corpuscles can be determined from the equation, diameter $= \frac{2{\cdot}402 \times 10^3}{\sqrt{CD}}$, where $C$ = number of cells per cmm. and $D$ = opacimeter reading. The diameter of the corpuscle is inversely proportional to the square root of the product of the cell count and opacimeter reading.

6. The opacity constant $k$ for any given mammalian red corpuscle, that is, the theoretical turbidity produced by one such corpuscle per cubic millimetre of suspension, can be determined from the equation $k = 0{\cdot}171d^2 \times 10^{-6}$, where $d$ is the diameter of the corpuscle.

7. The opacity of a suspension of red corpuscles is a measure of the total surface of the suspended particles.

8. In those physiological and pathological reactions where surface action plays a predominating part, opacity determinations offer a more strict measure of the changes taking place than number of particles.

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# XXVIII. THE RELATION BETWEEN THE MICROSCOPIC APPEARANCE OF PRECIPITATED CALCIUM OXALATE AND THE OPACITY OF ITS SUSPENSIONS.

By J. HOLKER.

*From the Department of Pathology, The University, Manchester.*

(*Received February 18th, 1921.*)

In the course of an investigation of the opacity of suspensions resulting from the complex interactions of electrolytes, suspensoids and emulsoids, it became advisable to study the factors governing the opacity of suspensions of relatively simple substances. It was decided therefore to begin with a crystalline organic substance, and for this purpose calcium oxalate seemed to be a suitable substance since by reason of its high degree of insolubility it offered a considerable range in the concentration of its suspensions. Moreover since the main research was concerned with organic substances capable of yielding insoluble products of hydrolysis, it was advisable to choose for the present investigation an organic acid of known chemical composition and crystalline appearance. It was thought that by comparing the microscopic appearance of the precipitate with the curve obtained by plotting the opacity of the suspension against the concentration, information would be gained which might be of use in studying the factors involved in producing the opacity of more complex suspensions. Such complex suspensions are the rule in serological investigations, and it seems to me that the problems of immunology will only be solved by approaching the subject from the physico-chemical standpoint. Then after analysing the simpler phenomena it should be possible to build up a framework on which to mould the more complex reactions.

The apparatus used for measuring opacity was that recently described by the author [1921,1]. The method of preparing the suspensions of precipitate was as follows. Into each of two dry test-tubes of resistance glass of about 80 cc. capacity were pipetted 25 cc. of known strengths of calcium chloride and potassium oxalate respectively. The two reagents were then thoroughly mixed by pouring a definite number of times from one tube into the other. The tube containing the 50 cc. of mixed reagent was then allowed to stand a predetermined time at room temperature so as to allow the turbidity to become constant.

The time necessary for attaining equilibrium was determined by plotting the opacity against the time. By this means it was found that in no case did

it take longer than half-an-hour for the opacity to become constant. With very dilute solutions however there was a lag before the precipitate appeared, and the more dilute the reagents, the longer the lag. This lag was no doubt due to the fact that in very dilute solutions the crystals are very small in size and very few in number. As is well known small particles of a precipitate dissolve more readily than large ones. For example, Hulett [1901] found that particles of calcium sulphate 0·0003 cm. in diameter had a solubility of 18·2 millimoles per litre, while with particles 0·002 cm. in diameter the solubility was 15·33 millimoles per litre. With more concentrated reagents the velocity of growth of the crystals became more and more rapid, and moreover there developed between the initial steep slope and the final level line of equilibrium a series of oscillations. The more concentrated were the reagents, the more pronounced became the oscillations.

In the next series of experiments 25 cc. each of equimolecular solutions of calcium chloride and potassium oxalate were mixed together in the manner described above, and, after allowing the mixture to stand at room temperature for half-an-hour until equilibrium was attained, the opacity was measured and plotted against the net concentration of the reagents. For instance, equal volumes of $N/200$ reagents produced a turbidity of 88; the latter figure was therefore plotted against a net concentration of $N/400$. Six or more experiments were made for each concentration of the reagents and the mean of them taken. At the same time the appearance of the precipitate under the high power with the microscope was examined, and the crystals drawn as nearly as possible to scale. The opacities of the equimolecular mixtures are plotted in Fig. 1, curve A. A summary of the drawings corresponding to these experiments is shown in Fig. 2.

According to the well-known law for the solubility product of a saturated solution of a difficultly soluble substance, excess of either of the ions of calcium oxalate will diminish the solubility of the calcium oxalate and so correspondingly increase the amount of the precipitate. It was decided first to test the effect of excess of potassium oxalate on the form of the curve.

For this purpose 25 cc. of $N/10$ potassium oxalate were mixed with 25 cc. of varying concentrations of calcium chloride from $N/10$ downwards. The results are plotted in Fig. 1, curve B. The microscopical results were similar in general character to those shown in Fig. 2, but differed in points of detail.

In the next series it was decided to test the effect of excess of calcium ions. For this purpose therefore 25 cc. of $N/10$ calcium chloride were mixed with 25 cc. of varying concentrations of potassium oxalate from decinormal downwards, the results being plotted in Fig. 1, curve C. The microscopical appearances were similar to those shown in Fig. 2.

It will be seen from Fig. 1 that the opacity concentration curves for the precipitation of calcium oxalate may be divided into three parts, viz. a short initial lag, a longer intermediate zone which increases in steepness to a maximum, and a very long final zone which decreases in steepness until the line

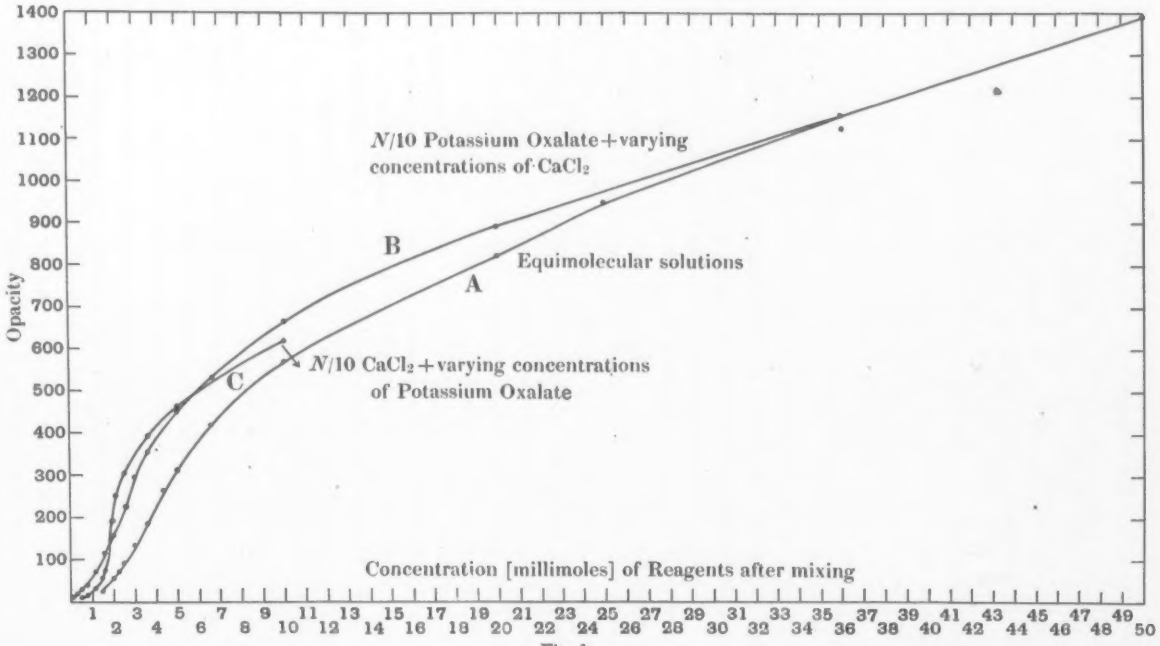
Opacity
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
N/10 Potassium Oxalate+varying concentrations of CaCl2
B
Equimolecular solutions
A
N/10 CaCl2+varying concentrations of Potassium Oxalate
C
Concentration [millimoles] of Reagents after mixing
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50

Fig. 1.

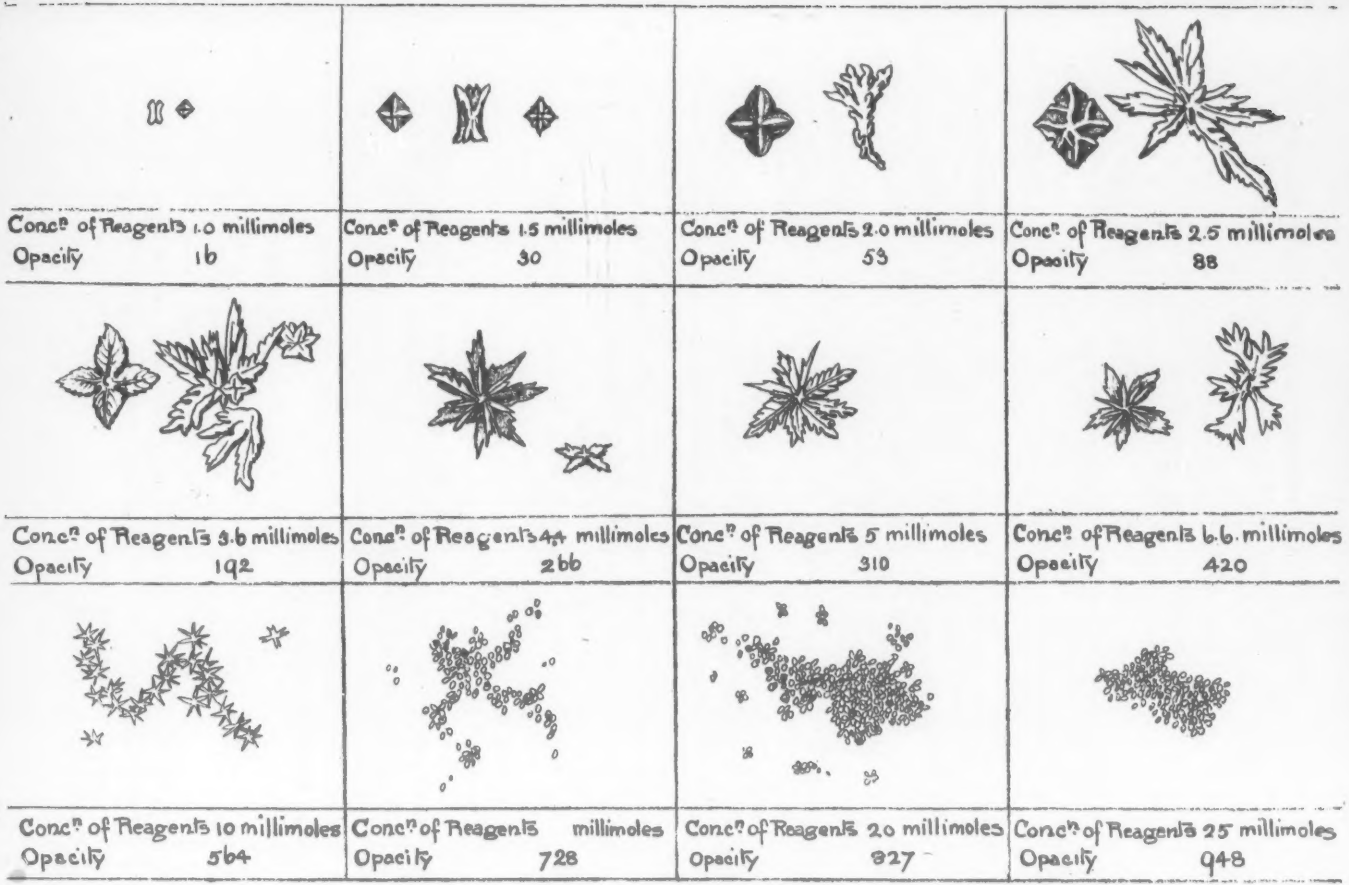


Fig. 2. Equimolecular solutions.

becomes nearly straight. As I have recently shown [1921, 2] that the opacimeter used measures the total surface of the particles scattering light, and that the opacity concentration curve is a straight line, in those cases where the particles remain all of the same size and shape in all concentrations, some explanation was required for the type of curve obtained with calcium oxalate. On studying the microscopic appearances of the precipitates corresponding to the zones in the curves, it was found that in the initial lag the crystals were very few in number and very small in size, and that they tended to be spherical in shape. The small number and size of the particles therefore accounted for the low turbidity in this zone. The ultimate cause of the low turbidity was no doubt the fact that small particles, having a relatively large surface, tend to redissolve in order to diminish the surface energy to a minimum. At a certain critical point in the curve represented by the beginning of the intermediate zone, the crystals begin to increase in size and number with increasing concentration of reagents. In this zone then the growth is mainly by surface condensation with the production of crystals of more or less typical structure. The growth in size goes on until the crystals have attained maximum size; this point being represented in the curve by a second critical point which marks the end of the intermediate zone and is accurately defined as the point of change in direction of the tangent to the curve. Immediately beyond the second critical point the crystals tend to be formed of spherical aggregates. With still higher concentrations of reagents the spherical aggregation becomes less pronounced and is finally replaced by a non-geometrical polar aggregation which becomes more and more pronounced. It is this increasing aggregation beyond the second critical point which is responsible for the continually decreasing steepness of the curve in the final zone.

The effect of excess of oxalate or calcium ions is well shown both in the curves and in the appearance of the precipitates. The curves bear out the solubility law in that excess of either oxalate or calcium ions leads to an increase in turbidity of the suspension. In the initial zone the lag with excess of oxalate ions is very short, while that with excess of calcium is intermediate in length. In the intermediate zone, the steepness with excess of calcium is very marked while that with oxalate is intermediate in degree. The maximum steepness or rate of increase of turbidity with respect to the concentration of the reagent is strikingly shown by the values for the tangents to the curves at the second critical points.

| | Maximum steepness of the curves |
|---|---|
| 1. Equimolecular solution ... | 1·32 |
| 2. Excess of oxalate ions ... | 1·70 |
| 3. Excess of calcium ions ... | 5·26 |

It would appear that excess of calcium ions is more favourable for the production of typical crystals of calcium oxalate, but even in this case there is a gradual transition in the type of crystal from the beginning to the end of the intermediate zone.

SUMMARY.

1. The turbidity of a suspension of calcium oxalate produced by the interaction of calcium chloride and potassium oxalate takes a certain time for the development of equilibrium.

2. With dilute solutions there is a lag before any turbidity appears.

3. With concentrated solutions there is a period of oscillations of the turbidity before equilibrium is established.

4. The turbidity concentration curve for equilibrium turbidities is not a straight line, but shows a short initial flat lag, a longer intermediate zone of increasing steepness, and a long final zone of diminishing steepness.

5. Two critical points in each of the curves represent the beginning and end of the intermediate zone. The first critical point represents the point at which the crystals have attained a size at which they do not tend to redissolve. It also represents the point of first appearance of the typical "envelope" crystal. The second critical point represents the point at which the typical crystals reach their maximum size. Beyond that point the crystals diminish in size but still increase in number. The second critical point also indicates the onset of aggregation of crystals, with consequent diminution in the rate of increase of turbidity.

6. Excess of either oxalate or calcium ions increases the opacity.

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# XXIX. THE OPACITY OF SERUM DILUTED WITH DISTILLED WATER, PHYSIOLOGICALLY NORMAL SALINE, AND RINGER'S SOLUTION.

By J. HOLKER.

*From the Department of Pathology, University, Manchester.*

(*Received February 18th, 1921.*)

As the author had recently been investigating the turbidity concentration relations of suspensions of the crystalline substance, calcium oxalate [1921, 3], and of suspensions of red corpuscles [1921, 2], he deemed it advisable to extend the work to the study of the emulsoid colloid, serum. In the routine of a pathological laboratory serum occupies an important position, forming, as it does, the basis of immunity reactions. For example, the Widal test for agglutinations by typhoid serum, and the Wassermann test for syphilitic serum, are reactions which are very frequently employed. Moreover it is customary to dilute the patient's serum with physiologically normal saline (0·85 % NaCl) with the object of keeping the osmotic pressure constant. In this way an impression has been created that, by keeping the osmotic pressure of serum constant, no other changes occur on dilution. The author has long doubted the correctness of this impression, and as a sensitive apparatus for measuring the opacity of liquids had been devised [1921, 1], it was decided to make use of this characteristic of colloids in the present investigation.

The general method employed was as follows. Serum was used which had been "inactivated" by heating at 56° for 20 minutes. From the inactivated serum was prepared a series of dilutions in Jena glass test-tubes, which were then allowed to stand for four hours at 40°, but were shaken every hour. It was found by preliminary experiments that four hours were sufficient for the opacity to become constant. In order to differentiate between normal and pathological conditions, sera were taken which were negative or positive to the Wassermann reaction.

In the first series of experiments, negative serum was diluted with distilled water, physiologically normal saline, normal saline plus calcium chloride, and Ringer's solution. Most of the results are plotted in Fig. 1, and the remainder, for dilutions less than 1/7·5, are given in Table I.

Table I.

The opacity of *negative* serum diluted with:
(1) distilled water,
(2) physiologically normal saline,
(3) physiologically normal saline plus 0·022 % calcium chloride,
(4) Ringer's solution (NaCl 0·85 %, $CaCl_2$ 0·022 %, KCl 0·056 %).

| | | Opacity of dilutions of serum | | | |
|---|---|---|---|---|---|
| Dilution of serum | Concentration of serum | Distilled water | NaCl 0·85 % | NaCl 0·85 % $CaCl_2$ 0·022 | NaCl 0·85 % $CaCl_2$ 0·022 KCl 0·056 |
| Undiluted | 100 % | 42 | 42 | 42 | 42 |
| 1/1·5 | 66·6 | 40 | ... | 50 | 53 |
| 1/2 | 50·0 | 37 | 28 | 46 | 50 |
| 1/3 | 33·3 | 35 | 27 | 43 | 47 |
| 1/4 | 25·0 | 33 | 26 | 39 | 44 |
| 1/5 | 20·0 | 32 | 25 | 37 | 42 |

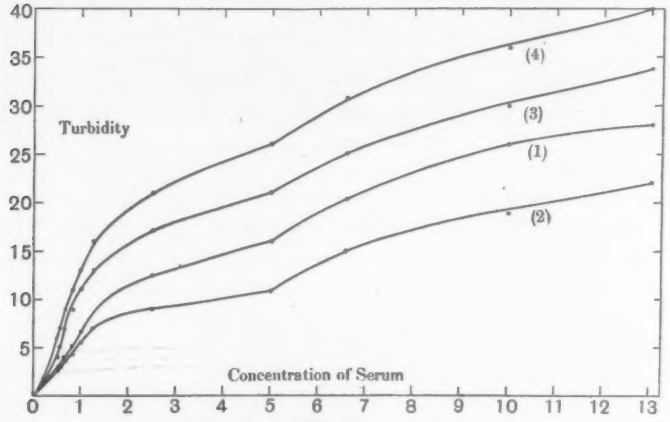


Fig. 1.

In the second series of experiments a pathological serum was taken which gave a positive reaction to the Wassermann test of + 1/10 but delayed lysis when diluted 1/20. The results are given partly in Table II, and partly in Fig. 2.

Table II.

The opacity of *positive* serum ( + 1/10, delayed lysis 1/20) diluted with:
(1) distilled water,
(2) physiologically normal saline,
(3) physiologically normal saline plus 0·022 % calcium chloride,
(4) Ringer's solution (NaCl 0·85 %, $CaCl_2$ 0·022 %, KCl 0·056 %).

| | | Opacity of dilutions of serum | | | |
|---|---|---|---|---|---|
| Dilution of serum | Concentration of serum | Distilled water | NaCl 0·85 % | NaCl 0·85 % $CaCl_2$ 0·022 | NaCl 0·85 % $CaCl_2$ 0·022 KCl 0·056 |
| Undiluted | 100 % | 50 | 50 | 50 | 50 |
| 1/1·5 | 66·6 | ... | ... | ... | ... |
| 1/2 | 50·0 | ... | 37 | ... | ... |
| 1/3 | 33·3 | ... | 35 | ... | ... |
| 1/4 | 25·0 | ... | 35 | ... | ... |
| 1/5 | 20·0 | 38 | 34 | 42 | 47 |

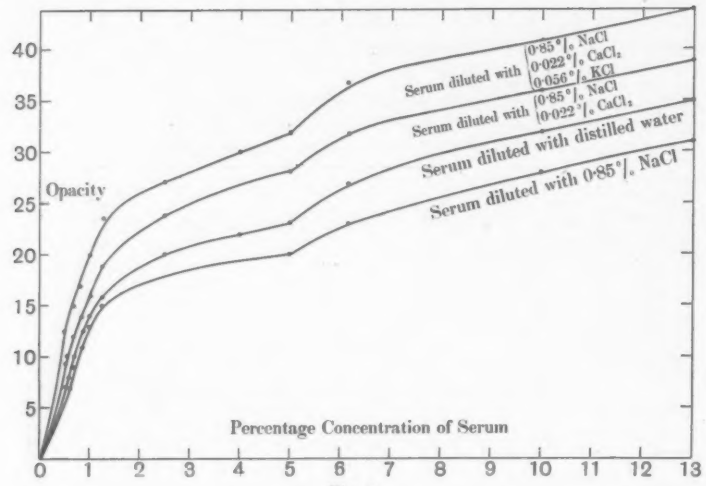
Opacity
Percentage Concentration of Serum
Serum diluted with 0·85 % NaCl 0·022 % CaCl2 0·056 % KCl
Serum diluted with 0·85 % NaCl 0·022 % CaCl2
Serum diluted with distilled water
Serum diluted with 0·85 % NaCl
0 1 2 3 4 5 6 7 8 9 10 11 12 13
5 10 15 20 25 30 35 40

Fig. 2.

In the third series of experiments a pathological serum was taken which gave a strongly positive reaction to the Wassermann test of + 1/40. The results are collected together in Table III.

Table III.

The opacity of a strongly positive serum (+1/40) diluted with:
(1) distilled water,
(2) physiologically normal saline,
(3) physiologically normal saline plus 0·022 % calcium chloride,
(4) Ringer's solution (NaCl 0·85 %, $CaCl_2$ 0·022 %, KCl 0·056 %).

| Dilution of serum | Concentration of serum | Opacity of dilutions of serum: Distilled water | NaCl 0·85 % | NaCl 0·85 % $CaCl_2$ 0·022 | NaCl 0·85 % $CaCl_2$ 0·022 KCl 0·056 % |
|---|---|---|---|---|---|
| 1/15 | 6·6 % | 40 | 33 | 48 | 53 |
| 1/20 | 5·0 | 36 | 29 | 44 | 48 |
| 1/25 | 4·0 | 35 | 28 | 43 | 47 |

It will be seen that whether the serum is normal or pathological, or whether it is diluted with distilled water or with salt solution, the turbidity concentration curve is not a straight line such as is produced in diluting a suspension of red corpuscles, but that for all dilutions the turbidity is greater than that which would be produced if the protein particles scattering the light had remained the same in size and had merely been proportionally reduced in number. For example, if the turbidity of the negative serum had varied directly with the concentration, then a dilution of ten times would have given an opacity of 42/10 = 4·2. But with distilled water the opacity was 26, with "normal" saline it was 19, with normal saline plus calcium chloride 30, and

with Ringer's solution, 36. Hence dilution of serum leads to an increase of the surface of particles scattering light, due probably to an increase both in the size and number of the particles. To account for the relative increase in surface of the protein particles of serum on dilution, it would be better first to endeavour to form some idea of the physico-chemical condition of the protein. Proteins are for the most part amino-derivatives of fatty acids. Hence their salts may be considered as amino-derivatives of soaps. Hence also their salts will exhibit the phenomenon of hydrolysis. At all dilutions of serum therefore there will be an equilibrium between the hydrolysed and the non-hydrolysed portions of the salts and the degree of hydrolysis will increase with dilution and with temperature. Moreover as serum is slightly alkaline in reaction, only minute traces of free acid can exist as such in the products of hydrolysis, and the insoluble portion of the protein must therefore be of the nature of an acid soap. The proteins of diluted serum therefore exist probably in the following forms: (1) non-hydrolysed protein salts, of which those with sodium are soluble, while those containing calcium are soluble, if the acid is derived from a lower fatty acid such as propionic acid, but insoluble if the acid comes from the higher group; (2) hydrolysis products which are acid in character, though combined with a certain amount of sodium for the most part, and with calcium to a slight extent, in both cases the higher products being insoluble and tending to form aggregates; and (3) a minute quantity of free amino-acid which will ionise with the production of (4) a very minute quantity of amino-acid anions. It seems therefore that when serum is diluted, hydrolysis of the protein salts occurs with the consequent relative increase in the particles scattering light. It will be seen in referring to Table I and Fig. 1, that the turbidity of small dilutions of serum is even greater than that of the undiluted serum. Such a phenomenon also occurs in the hydrolysis of sodium palmitate, in which the hydrolysis alkalinity first rises to a maximum and then falls away on further dilution. On referring to the curves it will be seen that at a dilution of 1/20 (5 % concentration) there is a definite discontinuity in all the curves, which is most marked with distilled water, and least marked with Ringer's solution. The significance of this discontinuity seems to be that in serum there are two distinct types of protein, one of which only begins to produce insoluble particles at dilutions below 1/20. Possibly these two types are represented by globulin and albumin.

The action of the ions of sodium, calcium and potassium is well shown in the curves. With 0·85 % sodium chloride not only is hydrolysis thrown back, but the non-hydrolysed portion of the salts is soluble. With "normal" saline plus 0·022 % calcium chloride, the insoluble products are increased because the calcium salts are insoluble. With Ringer's solution the products are still further increased. These antagonistic actions of sodium and calcium are comparable to the results obtained by Ringer on perfusing the excised heart. With "normal" saline the heart continued to beat feebly for some time, and then stopped in a condition of relaxation. On adding a trace of calcium chloride

the heart commenced to beat again, but more strongly, then stopped in a condition of tonic contraction. On further adding a trace of potassium chloride the heart beat much more strongly for many hours. In view of the above results with serum, it would appear that when the heart stopped under perfusion with saline the colloids of the cardiac muscle fibres contained relatively few insoluble particles. On the other hand when the calcium chloride was added the number of insoluble particles was increased, and finally, when the potassium chloride was added the number of insoluble particles was increased still further to a point corresponding to the condition found in the cardiac muscle of the living animal.

With regard to the pathological side of the investigation, it will be seen that syphilitic serum contains more insoluble matter than normal serum, and that the stronger the Wassermann reaction the greater is the quantity of insoluble matter. Correspondingly, of course, there will also be an increase in the "soluble" portion of the protein in syphilitic sera. It may be pointed out here that the strength of the Wassermann reaction is measured in practice by the greatest dilution to which the serum can be carried and still prevent the specific haemolysis of red corpuscles. For example the + 1/40 serum used in the present investigation ceased to prevent complete haemolysis after a dilution of 1/40, while the + 1/10 serum failed to prevent complete haemolysis beyond a dilution of 1/10. At a dilution of 1/40 (2·5 %) in "normal" saline, the strongly positive serum had a turbidity of 27. At a dilution of 1/10 (10 %) in normal saline the weaker positive serum had a turbidity of 28. But it was shown [1921, 2] that turbidity is a measure of the surface of the particles scattering light. Therefore the available adsorptive surfaces of the sera at their respective greatest dilutions were the same. This result therefore provides evidence for the theory that the Wassermann reaction depends on surface action.

In serological investigations the serum, after dilution and mixing with the antigen, is allowed to stand for some time at a definite temperature. It was advisable therefore to test the effect of change of temperature on the opacity. For this purpose negative serum was diluted five times with distilled water, with physiologically normal saline, and with Ringer's solution, and then allowed to stand in an incubator at different temperatures for four hours. The following were the results.

Table IV.

The effect of temperature on the opacity of negative serum diluted five times with:
(1) distilled water,
(2) physiologically normal saline (0·85 % NaCl), and
(3) Ringer's solution (NaCl 0·85 %, $CaCl_2$ 0·022 %, KCl 0·056 %).

| | Opacity of diluted serum | | |
|---|---|---|---|
| Temperature | Distilled water | NaCl 0·85 % | Ringer's solution |
| 5° C. | 22 | 22 | 22 |
| 15 | 24 | 23 | 25 |
| 24 | 28 | 24 | 30 |
| 30 | 30 | 24 | 31 |
| 40 | 32 | 25·5 | 33 |
| 57 | 32 | 27 | 35 |

The results if plotted show that the temperature curve for the limits 5° to 57° is a straight line in the case of physiologically normal saline and that the total effect is slight, increasing only from 22 at 5° to 27 at 57°. The slow increase in turbidity of diluted serum with increase of temperature is probably due to the increased solubility of the hydrolysis products at higher temperatures. On the other hand with both distilled water and Ringer's solution, the curve shows an initial lag up to 10° and then rises somewhat sharply up to 30°. Beyond 30° the curves are nearly flat. In the case of dilutions with distilled water, there is a progressive diminution in the quantity of salts present, with a consequent increase in the quantity of acid hydrolysis products, which are only slightly more soluble at higher temperatures. In the case of dilutions with Ringer's solution, the small quantity of calcium present (0·022%) is more than sufficient to counterbalance the effect of the sodium. The action of calcium in this case can scarcely be accounted for by its bivalency. It seems more probable that it is rather a question of decreased solubility of the calcium salts. Such a phenomenon is also observed with caseinogen salts, the sodium salts being soluble and the calcium salts relatively insoluble.

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# XXX. ON THE COMPOSITION OF THE UNSAPONIFIABLE MATTER OF THE ETHER EXTRACT OF HUMAN FAECES.

By JOHN ADDYMAN GARDNER.

*From the Physiological Laboratory, University of London, South Kensington.*

*Report to the Medical Research Committee.*

(*Received February 28th, 1921.*)

In a series of papers [1907–1912] on the origin and destiny of cholesterol in the animal organism, it was shown that cholesterol is never excreted as such, or in any recognisable reduced form, in the *normal* faeces of herbivorous animals, such as horses, cattle, sheep and rabbits. Evidence was also brought forward which lent support to the view that in herbivora cholesterol is a substance which is strictly conserved in the animal economy, that when the destruction of the red blood corpuscles, and possibly other cells, takes place in the liver, their cholesterol is excreted in the bile and that the cholesterol of the bile is re-absorbed in the intestine along with the bile salts, finding its way into the blood stream to be used again in cell anabolism; and, further, that any waste of cholesterol might be made up from that taken in with the food [Dorée and Gardner, 1909]. This latter process would be limited in herbivorous animals by the fact that their normal food does not contain cholesterol, but isomeric substances, such as phytosterols, which presumably would have to be converted into cholesterol before utilisation [Fraser and Gardner, 1910]. The faeces of herbivora, however, contain a considerable amount of non-crystalline unsaponifiable matter, consisting mainly of a reddish oil. The dry faeces of cows gave, after separating any crystalline matter, 0·39 % and of sheep 0·4 % of a red oil. 3 kilos. of dry summer faeces of wild rabbits yielded 0·21 % of so-called hippocoprosterol and 0·77 % of oil [Dorée and Gardner, 1908, 1].

It was found that this oil (from rabbits) was very stable and could be fractionally distilled at a pressure of under 1 mm. without any decomposition, though the process was tedious and difficult. Four main fractions were obtained at the following temperatures: (1) 98°, (2) 164–168°, (3) 215–220°, (4) 260–265°. In the flask there remained a transparent brittle resinous substance, which was still distilling without a trace of decomposition at the softening point of glass.

Fraction (1) was a pale yellow fairly mobile oil, with a smell recalling that of pine oil, which contained 82·13 % C and 11·96 % H. On treating a small quantity in chloroform solution with excess of acetic anhydride and a few drops of strong sulphuric acid, it gave a deep reddish brown colour, which gradually changed to a dirty brownish green. Fraction (2) had a faint pine odour and gave a similar reaction. Fraction (3) was a thick oil which gave the cholesterol colour reactions with the above reagents in a modified manner. Fraction (4)—the largest fraction—was at ordinary temperatures a clear yellow glass, which remained perfectly transparent after standing for ten years. It gave the sterol colour reactions with acetic anhydride and sulphuric acid in a well-marked though modified manner. Combustion showed that it had nearly the same composition as cholesterol.

| | % C | % H |
|---|---|---|
| | 84·21 | 11·56 |
| | 84·05 | 11·99 |
| Calculated for $C_{27}H_{46}O$ ... | 83·94 | 11·92 |

All attempts to prepare a crystalline acetate or benzoate failed.

The faeces of rabbits fed on ether-extracted grass also contained similar oils, but quantitative experiments to show whether in less quantity were not made.

Oils which gave a dirty brown or brownish green colour with acetic anhydride and sulphuric acid were also found in the unsaponifiable matter of the ether extracts of grass and other plants.

In carnivora, such as dog [Dorée and Gardner, 1908, 2] and cat [Ellis and Gardner, 1909], provided the body weight remains constant, the cholesterol excreted in the faeces can be all, or nearly all, accounted for by that naturally ingested with the food. Klein [1910], in his experiments, also arrived at a similar conclusion. In adult man, under normal conditions, cholesterol is not excreted as such in the faeces, but always in the form of one of the bihydrocholesterols. The main substance is coprosterol, but a certain amount of the isomeric $\beta$-cholesterol is also present.

Ellis and Gardner [1912] showed that the quantity of coprosterol found in the faeces of man can be largely accounted for by the cholesterol taken in with the food, provided the body weight remains constant. If, however, a rapid loss of weight takes place, as in illness, the output of cholesterol may largely exceed the intake. More recent experiments carried out by more accurate methods than were previously available, the results of which will form the subject of another paper, have shown that there is normally a regular though very small excretion of coprosterol in excess of the cholesterol taken in with the food. This would suggest that some organ of the body is capable of synthesising cholesterol to make up the waste. The unsaponifiable matter of human faeces contains in addition to crystalline sterols a considerable amount of non-crystallisable oils. These oils are somewhat variable in quantity, and often considerably exceed the amount of crystalline matter. After separa-

tion of coprosterol, etc. by means of digitonin they usually give the Burchardt-Liebermann reaction, though the colours are often partially masked by the dirty colour of the material. This colour reaction does not necessarily indicate that the oily matter contains derivatives of the sterol group, as the reaction is given by a variety of other substances.

It seemed, however, important to submit the unsaponifiable matter of faeces to a more detailed chemical examination.

For this purpose a large quantity—over 1 cwt.—of mixed human faeces from a Yorkshire village was collected and dried partly by heat and partly by means of plaster of Paris. The dry material was ground up and extracted with ether in large metal extractors, arranged on the principle of the ordinary small Soxhlet's apparatus. The ethereal solution of the extract was saponified in the usual manner in the cold with a large excess of an alcoholic solution of sodium ethoxide. The precipitated soaps were filtered off and re-extracted with ether. The ethereal filtrates were then washed free from traces of soap and alcohol by repeatedly shaking with water. The ether was then distilled off and the oily unsaponifiable matter taken up in acetone. The acetone solution was allowed to crystallise fractionally and the process repeated several times in order to effect as complete a separation of crystalline matter from it as possible.

In view of the results mentioned above it seemed possible that both the crystalline and oily matter might be conveniently purified by distillation in superheated steam, or by distillation in a high vacuum, or a combination of both processes.

In order to test the validity of this method as far as possible, and to ascertain whether any intra-molecular change took place in the case of the crystalline sterols, the behaviour of the following substances was examined: cholesterol, coprosterol and $\psi$-coprosterol.

*Distillation of cholesterol.*

As it is well known that cholesterol can be distilled *in vacuo* unchanged, it was therefore only submitted to distillation in superheated steam.

Steam from a boiler was passed through a small spiral superheater and carried by a metal tube to the bottom of an ordinary distillation flask containing the cholesterol. This flask was well lagged and its delivery tube passed to the centre of a second distillation flask, which was connected with a long Liebig's condenser.

The cholesterol melted and then distilled over in the steam fairly rapidly. The greater portion condensed in the trap flask, where it remained in the molten condition, and the rest condensed in the condenser in the form of a solid white jelly-like emulsion. This solid emulsion never blocked the condenser, but from time to time partly under the influence of the pressure of the steam and partly of the running condensed water was forced from the condenser into the receiver in the form of candles. The emulsion could be readily

filtered and a good deal of the water separated by gentle pressure. The various distillates were recrystallised from alcohol. The small quantity of cholesterol remaining in the distillation flask was only slightly discoloured, and on recrystallisation from alcohol melted at 146–147°.

The cholesterol used melted at 147–148°, and had a specific rotary power $[\alpha]_D = -36{\cdot}39°$.

The fractions from the trap flask and the receiver after recrystallisation from alcohol both melted at 147–148°. They showed however a slight increase in rotation. The values determined were:

| | |
|---|---|
| Undistilled cholesterol ... ... ... | −36·39° |
| Cholesterol from solid emulsion ... ... | −37·81° |
| Cholesterol from trap flask ... ... | −39·50°; another determination −39·46° |
| Cholesterol from residue in distilling flask | −35·59°. |

The cholesterol from the trap flask was again recrystallised from 80 % alcohol and gave a value −41·4°.

The meaning of these variations has not been ascertained.

*Distillation of coprosterol.*

10 g. of fairly pure coprosterol were distilled at a pressure of about 1 mm. from a small retort heated by a metal bath. The material distilled over readily at 220–225°. The apparent temperature of distillation, however, appeared to depend to some extent on the temperature of the bath and the process was more of the nature of an evaporation than a true distillation. The distillate was very pale yellow in colour and solidified to a white mass. Only a faint trace of tarry matter remained in the retort. The distillate was crystallised from acetone. The main fraction on heating began to shrink together at 102° and ran in the tube at 107°. Another small crop was obtained on evaporating the mother liquors, which melted at 101–102°. The distilled coprosterol had a normal rotation and the acetate and benzoate normal properties. The following esters were also prepared and may be briefly described:

*Chloroacetate.* 5 g. coprosterol were dissolved in 10 cc. chloroform and warmed under a reflux with a slight excess of chloroacetyl chloride. The residue obtained on evaporating the solvent crystallised from ethyl acetate in needle-shaped crystals. On heating they began to soften at 143° and melted at 145–146°. The substance was only difficultly soluble in hot methyl and ethyl alcohols. It contained 7·93 % chlorine—theory 7·63, and its specific rotary power in chloroform was $[\alpha]_D^{18} = +20{\cdot}6°$.

*Bromoisovalerate.* This was prepared in a similar manner from bromo*iso*-valeryl bromide. It crystallised readily from a mixture of ethyl acetate and alcohol, or from a mixture of chloroform and alcohol. It melted at 103–104° and had $[\alpha]_D^{18} = +18{\cdot}47°$. It contained 14·67 % bromine—theory 14·52.

*Stearate.* This was prepared by boiling coprosterol in chloroform solution with a slight excess of stearyl chloride. It was almost insoluble in hot alcohol,

but crystallised readily from ethyl acetate or acetone. It melted at 69–70°, and on cooling showed a faint yellowish green play of colours at the solidifying point. $[\alpha]_D^{18} = +14{\cdot}02°$ (in chloroform).

*Analysis:* 82·21 % C, 12·46 % H; calculated for $C_{45}H_{82}O_2$ 82·82 % C, 12·27 % H.

*Palmitate.* This was prepared in a similar manner from palmityl chloride. It was only very slightly soluble in methyl alcohol, but could be readily crystallised from ethyl acetate or from acetone in glistening hexagonal plates. It melted at 65° and showed at its solidifying point a faint colour display—greenish blue, green and yellow.

*Laurate.* This was prepared from lauryl chloride in a similar manner. It was more soluble in alcohol than the homologous esters, and crystallised readily from ethyl acetate in glistening leaflets, which under the microscope appeared as flat hexagonal plates, rather like phytosterol in appearance. It softened at 58° and melted at 61–62°.

*Analysis:* 82·43 % C, 12·34 % H; calculated 82·11 % C, 12·28 % H.

On oxidation the distilled coprosterol gave the ketone coprostanone described by Dorée and Gardner [1908, 3] in normal manner.

The distillation of the coprosterol had evidently produced no change.

### *Distillation of ψ-coprosterol.*

The *ψ*-coprosterol was prepared in the usual manner by boiling coprosterol in amyl alcohol solution with sodium amylate, and subsequent elimination of unchanged coprosterol and *β*-cholestanol by digitonin. The *ψ*-coprosterol left in the filtrate from the insoluble digitonide was then purified by recrystallisation. In the mother liquors, after separating crystalline matter as completely as possible, there remained a small quantity of a stiff oil. This oil distilled in a vacuum of 1 mm. at about 210–220° without any decomposition. The distillate had a pale yellow colour and solidified to an amorphous glass, which remained perfectly transparent for several years. It gave the sterol colour reaction in chloroform solution with acetic anhydride and sulphuric acid in a well-marked manner.

*Analysis:* 83·97 % C, 11·65 % H; calculated for $C_{27}H_{46}O$ 83·94 % C, 11·92 % H.

It had therefore much the same composition as cholesterol or coprosterol.

The *ψ*-coprosterol was then distilled in superheated steam when it passed over in a similar manner to the isomer. After crystallisation it was dried and submitted to distillation in a vacuum of 1 mm. pressure. It evaporated over between 214–222° according to the temperature of the metal bath, leaving no residue. It solidified in the receiver almost at once. The solid was recrystallised from alcohol, acetone, light petroleum and finally from alcohol and ethyl acetate. On heating it began to shrink together at 120° and ran in the tube at 123° to a turbid liquid which cleared at 125°. On cooling it became cloudy

again and was quite solid at 120°. On remelting it behaved as before. This melting point is rather higher than that given by Dorée and Gardner [1908, 3] 119° and by Windaus [1916] 116–118°. It is difficult to say whether the distillation had resulted in higher purification, or some slight change had taken place. The rotary power was found to be $[\alpha]_D^{14} = +28{\cdot}84°$.

The constants hitherto recorded are: Dorée and Gardner + 31·55°, and Windaus + 31·62°. The esters did not crystallise so readily as those of coprosterol; a number were prepared, which had normal properties. The following, which we believe are new, are worth a brief description:

*ψ-Coprosterol chloroacetate.* This was prepared in a similar manner to the coprosterol chloroacetate. It crystallised in glistening plates or flat needles. M.P. 68·5–69°. On analysis it gave Cl 7·82 %; calculated 7·91 %.

*ψ-Coprosterol stearate* was prepared by heating one part ψ-coprosterol with 0·8 part stearyl chloride in chloroform solution. It was crystallised from a mixture of chloroform and alcohol, and also from ethyl acetate. M.P. 62–63°. $[\alpha]_D^{18} = +28{\cdot}89°$ (in chloroform).

*Analysis:* 82·83 % C, 12·73 % H; calculated 82·57 % C, 12·53 % H.

*ψ-Coprosterol laurate* was made in a similar manner from lauryl chloride. It was purified by crystallisation from a mixture of benzene and alcohol, melted at 38–39°, and had $[\alpha]_D^{18} = +28{\cdot}43°$.

*Analysis:* 82·43 % C, 12·23 % H; calculated 82·11 % C, 12·28 % H.

As distillation did not seem to have produced any material alteration in cholesterol and coprosterol, the crude crystalline matter was purified by distillation in superheated steam and subsequent distillation in a vacuum of 1 mm. or under. The white solid matter thus obtained was then subjected to an elaborate fractional crystallisation from acetone and from 80 % alcohol. The bulk proved to be pure coprosterol, various fractions melting between 100 and 108°. Smaller fractions were, however, obtained which melted at 111–112° and 112–113°. On repeatedly recrystallising these, a fraction was isolated which began to shrink together at 113° and melted at 115–116°.

Hans Fischer stated that the melting point of coprosterol could be raised to 112–115° by repeated crystallisation.

In order to find whether this was pure coprosterol or contained some isomer, it was heated in amyl alcohol solution with sodium amylate in the usual manner to convert coprosterol into ψ-coprosterol. The product was dissolved in alcohol, and an alcoholic solution of digitonin added, when a small quantity of an insoluble digitonide was precipitated. The bulk of the product remained in solution and yielded pure ψ-coprosterol. The insoluble digitonide was dissociated by heating in the vapour of boiling xylene, and after driving off the xylene in steam, a small quantity of crystalline sterol was obtained. This gave no colour reaction with acetic anhydride and sulphuric acid, and from the appearance of the crystals under the microscope appeared to be β-cholestanol. The quantity was too small, however, for satisfactory

identification. It was, however, probably $\beta$-cholestanól, as the substance was found in considerable quantity in the oily residue left after the crystallisation of the crude coprosterol. Since this work was commenced Windaus and Uibrig [1915] have obtained from crude coprosterol by repeated crystallisation from light petroleum a small amount of a more difficultly soluble substance of higher melting point than coprosterol. The melting point was, however, not sharp. This substance was found to be impure $\beta$-cholestanol, which they succeeded in separating from coprosterol by the digitonin method and characterising by means of its esters.

*Examination of the dark oil left after separating the bulk of the crystalline sterols.*

This oil, in suitable quantities, was distilled in superheated steam in the apparatus described above, except that in this case the trap flask was kept in a brine bath at a temperature a few degrees above 100°. The whole distilled over leaving only a slight carbonaceous residue. A portion condensed in the brine flask as a stiff oil, but the bulk passed over into the condenser where it formed a solid emulsion, which was forced out of the condenser by the combined action of the condensed water and the pressure of the steam in solid white candles. This solid emulsion persisted for a long time, but after some days generally separated into oil and water. This was more readily effected by means of ether.

The property this oil possesses of forming these stable solid emulsions suggests that the "sterol" portion of the "unsaponifiable matter" may play an important part in the formation of faeces.

The thick viscid oils which collected in the brine flask were crystallised from acetone. These crystalline fractions—*A*—are described later. The oils from the mother liquors were again distilled in superheated steam. The solid emulsions which collected in the receiver were taken up in ether; the ethereal solutions were then dried and the ether distilled off. The oil left was then distilled in a vacuum of about 1 mm. and roughly separated into two fractions. The lower boiling fraction came over on the paraffin bath up to about 160–170°—*B*.

The higher fraction came over on the metal bath, mainly between 200 and 260°, and constituted much the greater portion of the material. It was very difficult to distil, as it was very viscid and if slightly overheated had a tendency to bump and froth over. An ordinary distillation flask proved quite unsuitable, but after trying various forms of apparatus a retort-shaped vessel was found to be efficient, and bumping was prevented by having a spiral platinum wire at the bottom. The retort was immersed in a metal bath, the portion outside the metal being well lagged. In later experiments it was found better to heat the retort in a Lothar Meyer air bath instead of a metal bath. The bath was kept a few degrees above the boiling point of the fluid. The distillation was done very slowly to prevent bubbling over, and was really more of an evapora-

tion than a true distillation. This high boiling distillate—*C*—set on cooling to a clear pale yellow glass with a slight greenish fluorescence. It recalled in appearance some of the high boiling distillates of petroleum.

On long standing it began to crystallise, bacteria-like crystals gradually spreading through the entire mass, which became opaque.

*Examination of fraction A.*

The solid matter from acetone crystallised in matted needles, M.P. 92–95°, and was rather like impure coprosterol. On recrystallisation from absolute alcohol, however, glistening plates—rather like cholesterol—separated. The melting point, after recrystallising several times, was raised to 136–137°, but was not sharp and the substance began to soften at a lower temperature.

These crystals contained 5·35 % of moisture; calculated for $C_{27}H_{46}O+H_2O$, 4·45 %.

*Analysis of dried material:* 83·807 % C, 12·034 % H; calculated for $C_{27}H_{46}O$, 83·94 % C, 11·92 % H; for $C_{27}H_{48}O$, 83·505 % C, 12·370 % H.

100 g. of 96 % alcohol dissolved at 21° 0·844 g. of the crystals. The solubility was therefore less than that of cholesterol. The substance was practically inactive to polarised light. On treatment in ethereal solution with a solution of bromine in glacial acetic acid a crystalline precipitate was formed. This was filtered and washed first with strong acetic acid and finally with water. After drying *in vacuo* it melted at 115–116°, and contained 29 % of bromine (calculated for cholesterol dibromide, 29·3 %). The yield was however less than half the theoretical.

The acetate was prepared in the usual manner. It crystallised from alcohol in glistening thin plates or flat needles. It was only slightly soluble in cold but readily in hot alcohol. It melted with previous softening at 105–106°, but on cooling did not solidify again until 80°. The benzoate was made by the pyridine method. After crystallisation from alcohol it began to shrink together on heating at 107° and ran in the tube at 120° to a clear liquid. During the heating it showed a colour display intermediate between those of cholesterol benzoate and $\beta$-cholestanol benzoate. After repeated recrystallisation from alcohol the product became more like $\beta$-cholestanol benzoate, and on heating coagulated at about 130° with a bright colour display, emerald green, blue and red according to the point of view. The colours vanished sharply at 157°, a clear colourless liquid remaining in the tube. On cooling the colour display appeared again at 157° and continued after the point of solidification. On mixing with some $\beta$-cholestanol benzoate the phenomena were unchanged. From these properties the inactive crystals appeared to be a mixture of approximately equal parts of cholesterol and $\beta$-cholestanol. In order to prove this the remaining substance was precipitated in ethereal solution with half the theoretical quantity of a solution of bromine in glacial acetic acid that would have been required had the substance consisted only

of cholesterol. After standing in an ice bath the crystals were filtered on the pump. The ethereal filtrate was poured into water and the solid recrystallised twice from alcohol. It was identified as $\beta$-cholestanol by mixed melting and the properties of its acetate and benzoate.

The dibromide was reduced by zinc dust and glacial acetic acid, and the product after hydrolysis with alcoholic potash was recrystallised from alcohol. It consisted of pure cholesterol.

This conclusion was also confirmed by the Liebermann reaction. $\beta$-Cholestanol does not give any colour reaction with acetic anhydride and a drop of sulphuric acid, or at any rate reacts only slowly and very slightly under the conditions in which cholesterol gives its typical reaction. The cholesterol was therefore estimated in the crystals by the method of Grigaut [1913] and they were found to contain 63·01 %.

*Examination of fraction B.*

This fraction consisted of a yellowish waxy solid, which separated from acetone as a white wax smelling of new mown hay. It was very volatile in superheated steam and readily distilled *in vacuo*. The material was submitted to an elaborate fractionation in a high vacuum. This proved rather difficult as it was not easy to keep the pressure quite constant. Ultimately the following fractions were obtained at about 1 mm.: (1) 112–118° (4 cc.); (2) 120–140° (3·75 cc.); (3) 140–150° (about 5·5 cc. mostly round 145°); (4) 155–165° (3 cc.); and about 6 cc. of oil above 200°, which was added to the main fraction *C*.

The first fraction was a white wax which, on treatment in chloroform solution with acetic anhydride and a drop or two of sulphuric acid, gave a reddish brown solution. The colour deepened on standing, and was very similar to the colour obtained with ferric chloride and an acetate. No change in colour took place on standing. The fraction 140–150° behaved similarly, but the higher fractions gave the sterol colour changes, *i.e.* the reddish brown gradually changed to green.

As it did not prove possible by distillation, at any rate with the quantities of material available, to isolate any pure products, the low boiling fractions which came over at 100–140° according to the pressure were dissolved together in a few cc. of alcohol, and the strong solution cooled in a good freezing mixture. With a suitable amount of alcohol the solution set to a sloppy crystalline mass. If it set too solid more alcohol was added. The crystals were then filtered by means of the pump through a well cooled filter. The crystalline matter on the filter was then recrystallised from acetone in which it was rather less soluble than in alcohol. It crystallised in leaf-like crystals which melted at 49–50°. The mother liquors on standing *in vacuo* set to a crystalline mass mixed with oil. This was freed from oil by spreading on porous tile, and on recrystallisation from alcohol a further crop of the above substance was obtained. In this manner several grams of crystals, M.P. 49–50°, were isolated. They proved

to be cetyl alcohol, and on mixing with a pure specimen of this substance showed no reduction in melting point.

*Analysis:* 79·31 % C, 14·05 % H; calculated for $C_{16}H_{34}O$, 79·34 % C, 14·05 % H.

On oxidation in glacial acetic solution by means of chromic acid, and also by heating to 250° with potash lime, it yielded palmitic acid, M.P. 62–63°.

0·1849 g. required for neutralisation 7·03 cc. *N*/10 soda; calculated for palmitic acid, 7·23.

The silver salt contained 29·15 % Ag; calculated for $C_{16}H_{31}O_2Ag$, 29·71 %.

A small amount of crystalline matter, M.P. 55–56°, was also isolated, but in too small quantity for purification. It was evidently a mixture of cetyl alcohol with some higher alcohol, and on oxidation yielded a mixture of acids.

*Examination of fraction C.*

This was separated into a number of fractions by distillation under a pressure of about 1 mm., but several on standing became opaque through formation of crystals, and evidently contained some coprosterol or other bihydrocholesterol. The various fractions were therefore dissolved in alcohol and separately precipitated by an excess of an alcoholic solution of digitonin. After standing the alcohol was evaporated at a low temperature and the oil separated from the insoluble digitonides and excess of digitonin by means of ether. After distilling off the ether the oils were again submitted to an elaborate process of fractionation in a vacuum of about 1 mm. This operation, particularly in the case of the higher boiling portions, was difficult and tedious. The difficulty in getting apparatus made during the years 1915–1919 rendered it necessary to work on a small scale, and to work up the material in comparatively small portions. The oils were also very viscous and showed a marked tendency to froth over, so that it was necessary to carry out the distillation very slowly and keep the temperature of the metal or air bath only a few degrees above the temperature of the distilling fluid. It was also found desirable to have the retort completely immersed in the bath. The process was therefore more of the nature of an evaporation than an ordinary distillation. It was also found difficult to keep the pressure constant within a millimetre or two with the pump available. Small variations at these low pressures have an appreciable effect on the boiling point. The apparent temperatures of distillation seemed also to be affected by the temperature of the bath.

Eventually the following fractions were obtained:

(1) 134–140°. This was a waxy material which was found to be similar to the cetyl wax described under *B*.

(2) Small intermediate fractions—155–165°, 165–175° and 175–200°.

(3) The main bulk distilled between 198–210°. It was obtained in various experiments in a series of fractions—198–200°, 202–204°, 199–202°, 202–210°,

200–205°. These were evidently the same substance, the differences in temperature range being probably due to slight unavoidable differences in conditions during the different distillations.

(4) A higher fraction, smaller in quantity—215–225°.

The fractions (3) and (4) all came over as pale yellow clear oils, having a faint greenish fluorescence.

They set on cooling to a clear glass, melting at 16–18°, and gave the sterol colour reactions with acetic anhydride and a little sulphuric acid in a well-marked though modified manner.

On analysis the various fractions gave the following results:

| | | % C | % H |
|---|---|---|---|
| No. 2. | 155–165°. Thick oil ... | 82·13 | 12·83 |
| | 165–175° ... ... ... | 83·03 | 12·17 |
| No. 3. | 198–210° ... ... ... | 84·81 | 12·19 |
| | 200–206° ... ... ... | 85·48 | 12·40 |
| | 199–202° ... ... ... | 84·49 | 12·40 |
| | 202–210° ... ... ... | 83·70 | 12·36 |
| No. 4. | 215–225° ... ... ... | 82·97 | 12·15 |

Determinations of molecular weight by depression of the freezing point of benzene, in the case of a fraction 200–206°, gave values of 355 and 395; calculated for $C_{27}H_{48}O$, 388.

In the case of substances of such high molecular weight and high carbon content, it is difficult to select a formula from the results of combustion. The figures for the fraction 202–210°, however, agree closely with those for cholesterol and bihydrocholesterol.

| | % C | % H |
|---|---|---|
| Calculated for $C_{27}H_{46}O$ ... | 83·94 | 11·92 |
| ,, $C_{27}H_{48}O$ ... | 83·51 | 12·37 |

This particular fraction remained as a transparent glass for several years. The other glassy fractions, however, on standing for some months gradually became more or less opaque owing to gradual separation of crystalline matter, and were very similar in appearance to bacterial growths in gelatin.

From these results it seemed probable that the fractions above 200° consisted of an amorphous substance of the sterol group mixed with more or less of some hydrocarbon or wax of very high molecular weight.

Attempts to separate crystalline matter by crystallisation from solvents in the ordinary manner were unsuccessful, but it was found possible to separate small quantities of crystalline matter by cooling strong solutions in acetone in a freezing mixture. All the fractions were therefore repeatedly treated in this manner. The waxy solids were too small in quantity for further treatment or identification, but the oils left after evaporating the acetone remained clear on long standing. They were redistilled in a vacuum of about 1 mm., and came over between 200–230° according to the pressure. They were all colourless pale yellow oils which set to glass on cooling. This glass melted about 16–17° to a very viscid oil.

On analysis the following results were obtained:

| | % C | % H |
|---|---|---|
| (1) 212–230° (1 mm.) ... | 83·12 | 12·56 |
| (2) 214–218° „ ... | 83·11 | 12·19 |
| (3) 202–216° „ ... | 84·04 | 12·18 |
| (4) 204–220° „ ... | 83·45 | 12·09 |
| (5) 214–225° „ ... | 84·16 | 12·21 |
| (6) 230–240° (2 mm.) ... | 83·75 | 12·01 |
| (7) 215–224° (1 mm.) ... | 84·04 | 12·23 |

From the variations in boiling point and in the figures by combustion it is evident that this oil is not a pure substance. It is impossible to deduce a formula from the figures. They might indicate anything from $C_{24}H_{42}O$, which requires 83·24 % C, 12·14 % H, to $C_{32}H_{58}O$, 83·84 % C, 12·66 % H, or, if the substance is unsaturated, from $C_{24}H_{40}O$, 83·72 % C, 11·63 % H to $C_{32}H_{56}O$, 84·21 % C, 12·28 % H.

From the boiling point, the colour reactions and great stability, it would seem probable that it may be a sterol of formula $C_{27}H_{48}O$ or $C_{27}H_{46}O$, or a mixture of a saturated and an unsaturated sterol.

Further details of the properties of this amorphous substance are given later on in this paper.

The digitonin precipitates, obtained from various portions of fraction *C*, were dissociated in vapour of boiling xylene, and the solid matter from the xylene was fractionally crystallised from acetone.

In this way, two fractions, one of indeterminate crystalline matter and the other in form of flat needles or spangles, were obtained.

The indeterminate crystals under the microscope appeared to consist of plates similar to crystals of $\beta$-cholestanol.

On heating in a capillary tube they began to soften at 122° and melted at 130–131°. The acetate of this substance was prepared in the usual way and crystallised from alcohol in shining leaflets, which on heating in a capillary tube began to soften at 120° and melted at about 124°, but the melting point was not sharp.

The benzoate was made by the pyridine method and after several recrystallisations from alcohol, in which it was difficultly soluble, showed the melting point and characteristic colour display of $\beta$-cholestanol benzoate. A mixed melting with $\beta$-cholestanol benzoate showed no depression of melting point, but rather more brilliant colours. Evidently the indeterminate crystals were mainly $\beta$-cholestanol. The needle-shaped crystals under the lens were very like phytosterol. They began to soften at 132° and melted at 136–137°. The acetate crystallised from alcohol in thin glistening leaflets, which on heating began to soften at 135° and melted sharply at 138–139°. The melted mass on cooling slowly crystallised in needle form. The substance did not form a benzoate by the pyridine method. The quantity was too small for further characterisation, but it was probably a phytosterol derived from vegetable food.

The results detailed above were obtained with mixed faeces—adults and children.

In the examination of the metabolism of a patient, suffering from anorexia nervosa, who was supposed to be on a specified diet, an extraordinarily large quantity of unsaponifiable matter was found in the faeces—40–50 g. in a week. This was distilled in a vacuum of 1 mm., and divided into a series of fractions ranging from 100–210°. These proved to be mainly paraffin hydrocarbons, and subsequent inquiry revealed the fact that owing to a misunderstanding the patient had been dosed with liquid paraffin up to the day on which the dietetic experiment commenced.

This result suggested that possibly the mixed faeces examined might have contained a certain amount of this material, which would contaminate the intermediate and higher fractions, and might account in the latter for the higher carbon and hydrogen content than that required for a true sterol.

It was therefore thought desirable to examine faeces from adults on rigid diet, and also from infants.

*Experiments with adults on known diet.*

For this purpose use was made of material accumulated in a series of experiments, on the digestibility of breads made from different kinds of flour, for a Report by the Food (War) Committee of the Royal Society [1918]. The subjects experimented on were for the most part laboratory attendants and assistants. The pre-arranged daily diet consisted of 800–1000 g. bread, 50 g. butter, 50 g. cheese, 50 g. minced meat, 100 g. of fruit jelly, 600 cc. milk, 30 g. sugar and tea or coffee *ad lib.*, though some individual minor variations were allowed. The subjects were fed for periods of ten or eleven days and the faeces of the last six used. The dried faeces, finely powdered, were extracted with ether, and the extract saponified in the manner already described.

The unsaponifiable matter was then crystallised from acetone as before to separate oil and crystalline matter as far as possible. In this case, however, the remaining oil was treated in alcohol solution with excess of digitonin to get rid of the remaining precipitable sterols before distillation.

The oil was then distilled in superheated steam, and the distillate fractionated in a high vacuum.

The oil was somewhat greater in quantity than the crystalline sterols. About 9 g. on distillation in superheated steam only left in the distilling flask 0·14 g. of carbonaceous matter.

The distillate proved rather difficult to fractionate in a high vacuum as traces of some decomposable matter were present and rendered it difficult to keep the pressure at 1 mm. In the initial distillation the following fractions were obtained:

(1) Up to 167° at 2–3 mm., but mostly round 127°. Wt. 0·49 g.
This was liquid and on standing deposited crystals.

(2) 177–187° at 2–3 mm. This was solid wax. Wt. 0·87 g.

(3) 187–197° at 5 mm. Wt. 0·68 g.

(4) To 225° at 5–6 mm. Wt. 0·83 g.

(5) 227–287° at 2–3 mm. Wt. 5·69 g.
This was a yellowish oil with a greenish fluorescence, which solidified to glass on cooling.

Residue left in flask, 0·38 g.

These fractions, beginning with No. 2, were now refractionated, and no difficulty was found in keeping the pressure at 1 mm. The following fractions were obtained:

| | | | | |
|---|---|---|---|---|
| (*a*) | Up to 137° at 1 mm. | — | | This solidified in the receiver as a white crystalline wax. |
| (*b*) | 130–147° | „ | — | This was solid, and did not give any colour with acetic anhydride and sulphuric acid. |
| (*c*) | 149–180° | „ | — | A viscid oil. |
| (*d*) | 187–211° | „ | Wt. 1·79 g. | This solidified to a transparent glass, which changed to a viscid pale yellow liquid at 20–22°. |
| (*e*) | 211–221° | „ | Wt. 2·58 g. | This was a pale yellow oil with a greenish fluorescence, which set to a glass on cooling. |
| (*f*) | 227–257° | „ | Wt. 0·72 g. | A clear oil with a greenish fluorescence, especially marked in chloroform solution. |

Fractions (*a*), (*b*) and (*c*) were dissolved in a little acetone and frozen. The solution set solid, but on filtering on the pump only a small amount remained unmelted. This was recrystallised from alcohol and identified as cetyl alcohol. From the acetone mother liquors a portion of a liquid nature was obtained. On heating this with potash lime hydrogen was evolved; 0·8 g. mixed with four times its weight of potash lime and heated at 230–250° gave 75 cc. of hydrogen at 25° and 760 mm. The residue was dissolved in hydrochloric acid and the fatty acids extracted with ether. The ethereal solution was extracted with aqueous potash, and the alkali solution again acidified and extracted with ether. On evaporating the ether a brown greasy acid was obtained. This was purified by distillation *in vacuo*, and proved to be a mixture of crystalline and oily acids, but too small in quantity for further separation. Evidently, however, the fractions (*a*), (*b*) and (*c*) contained cetyl and other alcohols of the paraffin series. The fractions (*d*), (*e*) and (*f*) all gave the Burchardt-Liebermann reaction in a modified though well-marked manner, and were quite similar to the corresponding fractions from the mixed faeces.

| *Analysis:* | % C | % H |
|---|---|---|
| (*d*) | 84·61 | 11·93 |
| (*e*) | 83·48 | 11·95 |
| (*f*) | 83·61 | 11·64 |

The crystalline sterols proved to be mainly coprosterol with a small amount of $\beta$-cholestanol.

The results of this investigation were therefore very similar to those obtained using mixed faeces. In 27 diet periods, during which four different kinds of bread were used, the rest of the diet being much the same, the ratio of the average amount of sterols precipitable by digitonin to unsaponifiable matter not so precipitated was 1 : 0·734. The ratios in individual cases were variable, ranging from 1 : 0·25 to 1 : 1·18, but in the majority of cases the individual ratios were not far from the mean value.

The ratio of the lower boiling portion, which did not give the Burchardt-Liebermann reaction, to the high boiling "sterol" portion which did, was about 1 : 4.

*Examination of infants' faeces.*

The faeces of healthy infants were examined in a similar manner.

In the case of breast fed healthy infants in the early weeks of life the crystalline sterol matter was found to consist entirely of cholesterol. The portion of the unsaponifiable matter not precipitated by digitonin was very variable in quantity in different individuals, and was usually considerably in excess of the cholesterol. This non-cholesterol matter has not yet been thoroughly examined, owing to the difficulty in obtaining a sufficient supply of digitonin, but it was found that in nearly all cases, after completely separating cholesterol by means of digitonin, the oil washed away from the insoluble digitonide by means of ether or light petroleum no longer gave either the normal or modified reaction with acetic anhydride and sulphuric acid. On treatment in chloroform solution with excess of acetic anhydride and a few drops of sulphuric acid a reddish brown colour developed but this did not change, and even on long standing no trace of green colour was noticed. On allowing to stand until the chloroform had evaporated spontaneously crystalline matter separated which could be isolated after dilution with water. Possibly this may afford a convenient method of purifying and identifying some of the constituents. A considerable amount of material from infants of various ages is being accumulated, and we hope to deal with this in another communication.

On the other hand, in pathological cases the amount of cholesterol is often less and the accompanying oils much greater in quantity than normal, and the latter show the acetic anhydride sulphuric colour reaction in the modified manner observed in the case of adult faeces. As an example we may quote the case of a prematurely born child (7 months), the fat metabolism of which was examined in some detail. It was brought into the hospital in the summer of 1915, when a few weeks old, suffering from bilateral conjunctivitis, and remained several months. Very little history of the case is available, but it was stated that its weight at birth was $3\frac{1}{2}$ lbs., and during its stay in hospital the stools according to the reports were apparently normal. After leaving the hospital the case was lost sight of, but subsequent inquiry elicited the information that "nothing of interest" developed and the infant gradually wasted away and died at the beginning of 1916, but no post mortem was made. During its stay in hospital a feeding experiment was done for seven days. It was fed on Allen and Hanbury's Infant Food No. 1, and weighed during the period 5·5 to 5·75 lbs. During the seven days it consumed 627 g. of the dry food, and excreted far more fat, measured as ether extract, than it ingested. The intake of unsaponifiable matter was 1·68 g. containing 0·36 g. of cholesterol, and the output in the faeces 25·5 g. of unsaponifiable matter containing 3·65 g. of cholesterol. The excess of output over intake was thus 23·8 g. of unsaponifiable matter and 3·29 g. of cholesterol, *i.e.* 3·4 g. and 0·47 g. respectively per day. The oil left after separation of the cholesterol digitonide gave the acetic anhydride sulphuric reaction in a well-marked manner.

The main bulk of the unsaponifiable matter was extracted first with acetone and then with alcohol, and the solutions allowed to deposit as much crystalline matter as possible. A small portion was insoluble or difficultly soluble in alcohol. This was dissolved in benzene and the solution diluted with ethyl acetate and left to crystallise. The mother liquors after separating as much crystalline matter as possible were evaporated and distilled in superheated steam. The great bulk distilled over as a solid emulsion, in the manner already described, leaving only a very small amount of carbonaceous residue. The distillate after separation from water was taken up in acetone and the solvent allowed to evaporate spontaneously, when the material gradually separated in oily form. It was collected in this manner in four fractions. Each fraction was then dissolved in acetone to a fairly concentrated solution and cooled in a good freezing mixture. In each case a small amount of wax solid at ordinary temperatures, a portion solid in the cold but fluid at laboratory temperature and an oil were obtained. These portions were then separately submitted to fractional distillation at a pressure of 1 mm. The fractions of similar range from different specimens were mixed and refractionated. In many cases the fractions were further purified by again freezing from acetone. In this manner the material was separated into (*a*) crystalline waxy solids, (*b*) a series of fractions which gave no colour with acetic anhydride and sulphuric acid, and (*c*) a series which gave with this reagent a reddish brown colour which more or less rapidly changed to bright green.

*Crystalline fractions* (*a*). From this crystalline matter about 1 g. of a substance insoluble in cold alcohol but soluble in hot was isolated. It separated from hot alcohol in gelatinous form. It was difficultly soluble in acetone but readily in benzene and in chloroform. It was readily crystallisable from benzene solution on addition of acetone. It melted sharply at 68–69°, and on cooling resolidified again at 67°, and remelted again at 68–69°. It was quite stable at 200° and could be distilled. It gave no trace of colour with acetic anhydride and a drop of sulphuric acid.

| *Analysis:* | % C | % H |
|---|---|---|
| | 82·52 | 13·90 |
| | 82·79 | 13·73 |
| $C_{30}H_{60}O$ requires | 82·57 | 13·76 |
| $C_{31}H_{62}O$ „ | 82·66 | 13·78 |
| $C_{32}H_{64}O$ „ | 82·76 | 13·79 |

Cholesterol was also isolated and identified by its crystalline form, melting point, dibromide and characteristic benzoate.

The white waxy materials, isolated from the fractions (*b*) by freezing from acetone, after recrystallisation from alcohol melted at various temperatures between 50 and 70°, but were too small in quantity for further separation and identification. One fraction, however, which melted at 49–50° was recognised as cetyl alcohol.

*Fractions of group (b) which did not give the sterol colour reaction.* These were (1) 190–198° at 1 mm., weight 0·37 g., (2) 198–204° at 1 mm. wt. 0·32 g.,

(3) 182–190° at 1 mm. wt. 0·52 g., (4) 190–212° at 1 mm. wt. 0·54 g., (5) 212–224° at 1 mm. wt. 0·33 g., (6) 208–230° at 1 mm. wt. 0·32 g.

These were all low melting white waxes, soluble in acetone, from which they separated in solid form on freezing.

No. (3) gave on analysis 85·62 % C, 13·54 % H.

This analysis, which adds up to 99·2, suggests a mixture of hydrocarbon and alcohol. Possibly the infant might have been dosed with liquid paraffin, but there was no mention of this in the records of the case.

A portion of these waxes was dissolved in glacial acetic acid and oxidised on the water bath with the requisite amount of chromic acid for one hour. The product was poured into water, the precipitate taken up in ether and the acids formed isolated in the usual manner. The product appeared to be a mixture of acids, but was too small in quantity for any attempt to separate and identify them. Titration in alcoholic solution gave an average molecular weight of 290.

Group (*c*), which gave the "sterol" colour reaction, contained the following fractions: (7) 167–169° at 1 mm. wt. 0·85 g., (8) 185–195° at 1 mm. wt. 1 g., (9) 192–208° at 1 mm. wt. 0·08 g., (10) 209–220° at 1 mm. wt. 0·5 g., (11) 207–210° at 1 mm. wt. 0·16 g., (12) 218–220° at 1 mm. wt. 0·43 g., (13) 212–230° at 1 mm. wt. 0·97 g., (14) 205–213° at 1 mm. wt. 0·4 g.

These all remained quite transparent on standing many months. Some other intermediate fractions however deposited crystals on standing, and these were separated and identified as cholesterol.

Fractions (7) and (8) differed from the rest in being at ordinary temperatures quite mobile yellow oils.

Fractions (7) and (8) were analysed:

| | % C | % H |
|---|---|---|
| (7) | 83·16 | 12·36 |
| (8) | 84·89 | 12·60 |

Both appeared to be unsaturated. No. (7) had an iodine value 49·0 (Wijs).

Both gave on treatment in chloroform solution with acetic anhydride and a drop or two of sulphuric acid a reddish brown colour, which in two or three minutes turned a dusky sage green and then bright green. This faded on standing over night to a brown.

As there might have been esters that had escaped hydrolysis in the original saponification, they were heated for some hours in sealed tubes with alcoholic potash at 110–120°. On cooling the material was recovered quantitatively and apparently unchanged. No acid was found on examining the alkaline mother liquors. On treating the recovered oil with digitonin no trace of precipitate was obtained.

The other fractions were at ordinary temperatures transparent pale yellow glasses, or else very viscous oils which would scarcely flow. They gave the sterol colour reaction in the modified way described above in a well-marked

manner, and appeared to be similar to the amorphous glassy substances obtained from the faeces of adults.

On combustion, fraction (13) gave 84·12 % C, 12·40 % H. Fraction (12) had an iodine value 73 (Wijs), fraction (10) 55·1 and another, 180–190°, 36·6.

*Examination of the faeces of soldiers in training.*

In order to ascertain whether cholesterol as such is excreted by the normal adult along with coprosterol and $\beta$-cholestanol, it was necessary to examine a large quantity of the excreta of adults on a more or less known diet.

Through the kindness of the D.D.M.S. of the Eastern Command and particularly of Lt.-Col. Jacomb Hood, I was able to obtain 51 stools of soldiers in training. They had the ordinary military ration of 1915, and were actively engaged in military duties.

This material yielded, on extraction in the usual manner, 110 g. of ether extract. This contained a considerable amount of free acid and required for neutralisation 7·23 g. of caustic soda, equivalent to about 51 g. of stearic acid. After saponification in the manner already described it yielded 26·93 g. of unsaponifiable matter as a dark brown oil. 0·6733 g. of this oil on precipitation with digitonin in alcoholic solution gave 1·1452 of compound equivalent to 0·2783 g. of sterol reckoned as coprosterol. The ratio of sterol precipitable by digitonin to the rest of the unsaponifiable matter was therefore 1 : 1·42, and the total unsaponifiable matter contained 11·13 g. of crystalline sterols—mainly coprosterol. The rest of the unsaponifiable matter was then treated with hot acetone, when it readily dissolved with the exception of a small amount of an insoluble crystalline substance. This weighed 0·6735 g. It was difficultly soluble in hot acetone, but soluble in boiling alcohol, from which it separated on cooling in a gelatinous form. It was readily soluble in benzene and chloroform, and could be crystallised from a mixture of benzene and ethyl acetate. It melted at 80–81°, and gave no colour with acetic anhydride and sulphuric acid. It was very similar to the substance isolated from the early born infant described above, but had a much higher melting point.

| *Analysis:* | % C | % H |
|---|---|---|
| | 82·53 | 14·68 |
| | 82·12 | 14·16 |
| Calculated for $C_{30}H_{62}O$ | 82·19 | 14·15 |

It seemed similar to the descriptions of myricyl alcohol. The acetone solution of the oil on standing deposited a rich crop of crystals of coprosterol, from which 9·1 g. of pure substance were obtained. The mother liquors by the digitonin method gave about 5 g. of insoluble compound which yielded about 1 g. of crystalline sterol. On fractionation from alcohol, 0·5 g. was obtained in the form of glistening leaflets and the rest in nodules of needles, mainly coprosterol. The leaflets were optically inactive, melted with previous softening at about 133° and were very like the similar material obtained from the Yorkshire

faeces. They were found by similar methods to consist of approximately equal parts of cholesterol and $\beta$-cholestanol.

Finally an artificial mixture of cholesterol and $\beta$-cholestanol in equal proportions was crystallised from alcohol and found to have properties closely resembling these crystals.

After separating the sterol-digitonide, 14 g. of brown oil partially dry was left. This was distilled in superheated steam, when it came over in the usual manner. 4·8 g. were carried over into the receiver in the form of a solid emulsion, 7·07 g. were caught in the trap flask, and only 0·2 g. of tarry residue remained in the distilling flask.

The oil from the emulsion was then distilled at about 1 mm. pressure, and the part distilling below 120° was dissolved in a little alcohol and cooled in a freezing mixture. In this way a small quantity of crystalline matter was obtained, which after purification melted at 49–50° and was identified as cetyl alcohol. The rest separated from acetone on freezing in solid form, but was difficult to filter. It was therefore added to the rest of the oil, which was then fractionated at a pressure of about 1 mm. first from a paraffin bath and then from a metal bath. 0·8 g. passed over below 150°, mostly round 120°; 1·48 g. above 150° on paraffin bath, and 7·5 g. above 200° on metal bath. The residue weighed 0·68 g. It was impossible to keep the pressure constant during this preliminary distillation owing to some slight amount of decomposable matter. On refractionation this difficulty was not encountered. Ultimately the following fractions were obtained at a pressure of about 1 mm.:

| | | | |
|---|---|---|---|
| (1) | 100–109° | Wt 0·2 g. | Liquid which solidified to wax on cooling. |
| (2) | 127–130° | „ 0·3 „ | Waxy solid. |
| (3) | 130–134° | „ 0·3 „ | „ |
| (4) | 136–140° | „ 0·11 „ | |
| (5) | 140–164° | „ 0·66 „ | Viscid oil, which partly crystallised on standing. |
| (6) | 164–190° | „ 0·45 „ | Clear liquid, solidifying to glass on cooling. |
| (7) | 190–202° | „ lost | |
| (8) | 202–209° | „ 1·4 „ | Pale amber coloured clear glass. |
| (9) | 209–230° | „ about 4 g. | This was distilled very slowly, and the temperature recorded by the thermometer seemed to vary with the temperature of the bath. It was an amber coloured glass at ordinary temperature. |

A small amber coloured residue was left in the flask.

The fractions (1) to (5) all gave in chloroform solution with acetic anhydride and a drop or two of sulphuric acid a reddish brown colour, which did not change on standing. The fractions (7) to (9) and also the residue on similar treatment gave a reddish brown colour which changed in a minute or two to a dusky green, and finally to a bright green. On standing over night this green gradually faded and became brown. Thus approximately about one fifth of the oil volatile in superheated steam consisted of alcohols which did not give sterol colours with acetic anhydride, *i.e.* about 8–9 % of the original unsaponifiable matter. Adding also the cetyl alcohol, this non-sterol portion would amount to about 11–12 % of the total unsaponifiable matter.

Fractions (8) and (9) were analysed and found to contain:

| | % C | % H |
|---|---|---|
| (8) | 84·29 | 12·10 |
| (9) | 84·73 | 11·95 |

*Properties of the amorphous glass distilling at* 200–230°.

The various fractions distilling at this range consisted of pale yellow or amber coloured amorphous glassy solids, melting at 16–18° to thick viscid liquids usually showing in bulk a slight greenish fluorescence.

The different distillates were not pure substances as is evident from the combustions. They appear to contain oxygen in a not very reactive form, and are marked by considerable stability. They give the sterol colour reactions in a modified way. When cholesterol in chloroform solution is treated with excess of acetic anhydride and a few drops of sulphuric acid, in general a pink colour first appears which rapidly changes to mauve, then violet, blue, blue green and finally green. The green stage is persistent for a long time, but gradually fades and after some hours a brown solution is left. With less quantities of sulphuric acid the initial pink and mauve stages often do not appear. Coprosterol under similar treatment gives a slate blue which deepens to blue and then passes through blue-green to a green similar to that of cholesterol. This slowly fades in a similar manner. $\psi$-Coprosterol gives much less marked colours and they take longer to appear. Generally a slate tint first appears and this changes to green, but the green is of a yellower type than that of cholesterol. $\beta$-Cholestanol when pure does not seem to give any colour reaction.

The amorphous bodies behave somewhat differently; on adding the sulphuric acid a deep reddish brown colour first appears, very similar to the colour of ferric acetate. In a few minutes this becomes a dusky sage green, which finally changes to a bright green. This stage persists for a long time. On standing some hours the green gradually fades and gives place to a brown. This green colour, and also that of $\psi$-coprosterol, is of a somewhat different quality to that given by cholesterol. The latter is a bluish type of green, the former a yellower type. This renders it rather difficult to compare the depth of colour of the one green with the other in a colorimeter.

The substance appeared to be a mixture of saturated and unsaturated sterols. Determinations were made of the iodine values of various samples by Wijs's method, but the results were very variable.

Fractions (*d*), (*e*) and (*f*) (p. 257) of the subjects fed on the bread diets were found to have values 62·5, 67·4 and 86·2 respectively. The theoretical value for cholesterol is 65·8. In case of specimens from the Yorkshire faeces (p. 255) lower values were obtained—No. 2, 31·1; No. 6, 29·7; No. 7, 43·1.

Other fractions gave 30·8, 30·8, 44·2, 31·1, 37·7, 57, 29·7 and 87·6.

Two specimens from infants' faeces gave 36·5 and 33·3. The substance obtained in the preparation of $\psi$-coprosterol (p. 248) had a value 37·5.

Much reliance cannot, however, be placed on these values since it has been shown by Smedley MacLean and Thomas [1920] that, for reasons not yet elucidated, Wijs's method gives abnormally high and variable values for cholesterol. This observation we can fully confirm.

Attempts to prepare crystalline or solid bromine addition products yielded negative results.

Attempts to determine the function of the oxygen in the substance also yielded inconclusive results. It had no reaction with hydroxylamine and other ketone reagents.

All attempts to prepare crystalline esters failed, though some indications that the substance was of an alcoholic nature—possibly tertiary—were obtained.

A specimen, B.P. 202–206°, was acetylated by means of acetic anhydride and sodium acetate and yielded a clear viscid oil. This could not be crystallised, but distilled in a high vacuum between 230–240° without apparent decomposition. With the Burchardt-Liebermann reagent it gave a reddish brown colour, which rapidly turned dusky green. On hydrolysis it yielded 7·46 % acetic acid; calculated for cholesteryl acetate 14·02 %. The unsaponifiable matter in the above experiment was recovered and again acetylated by prolonged boiling with acetic anhydride and sodium acetate. The product was not distilled, but freed from excess of acid by shaking the ethereal solution with alkali. On hydrolysis it now yielded 14·45 % acetic acid. Apparently therefore the substance had been acetylated.

On boiling with chloroacetyl chloride, hydrogen chloride was evolved, but the product was non-crystalline, and attempts to prepare a solid body by replacing the chlorine by a piperidyl residue failed. An amorphous product obtained by benzoylating a specimen with benzoyl chloride in pyridine solution at the boiling point, after being thoroughly freed from any free benzoic acid, was distilled in a high vacuum. It decomposed giving benzoic acid and an oil, the main portion of which distilled at 200–220° at 1 mm. This was a viscid amber oil at summer temperature which set to a glass on cooling, and gave the sterol colour reaction markedly in the modified manner. It contained 86·04 % C and 11·53 % H. Attempts to isolate a crystalline hydrocarbon from this, however, failed.

Other specimens gave analogous results, and probably the material consisted of an alcohol or mixture of saturated and unsaturated alcohols which was partially benzoylated under the conditions of experiment, and on distillation the benzoate decomposed into benzoic acid and a hydrocarbon, which was obtained in the higher fraction of the distillate along with original unchanged material.

In order to ascertain whether it would be possible to isomerise the material in a manner similar to the change of coprosterol to $\psi$-coprosterol and *vice versa*, and so possibly obtain a crystalline substance, 2 g. of a specimen, which distilled at about 200° and was a clear glass melting at 16–18°, were boiled with

20 cc. of amyl alcohol and 1 g. of sodium for seven hours. The amylic solution, after cooling, was washed first with water and then with acid. The amyl alcohol was then driven off in steam and the residue taken up in ether. Attempts to crystallise the substance from various solvents failed. The solutions on spontaneous evaporation all left a clear glass. It was therefore distilled at a pressure of 1 mm. with the bath only a little higher than the apparent temperature of distillation. It began to distil at 200°, passed over at much the same range as the original, and solidified in the receiver as a pale yellowish glass. It differed from the original in giving the sterol colour reactions in a typical well-marked manner—crimson, violet, blue and finally bright green.

*Analysis:* 84·42 % C, 12·26 % H.

The iodine value (Wijs) was found to be 30·5. Calculated for a compound of the carbon content found and one double link = 55·5 %.

On treatment with phosphorus pentachloride the oxygen was replaced by chlorine with evolution of some hydrogen chloride.

In one experiment 0·5 g. of the last fraction of the soldiers' material was dissolved in light petroleum and treated with phosphorus pentachloride in slight excess. The mixture was boiled under a reflux until everything went into solution and evolution of hydrogen chloride ceased. The light petroleum solution was then poured on to a large bulk of water and allowed to stand until the petroleum had all spontaneously evaporated. The water was poured off from the oil, which was again taken up in light petroleum, and the solution well washed with water, dried and evaporated at the ordinary temperature. In this way a thick oil was obtained with a chlorinaceous smell. As it seemed to darken when placed in the water oven it was dried *in vacuo* over sulphuric acid for several days and obtained as a pale yellowish brown oil.

*Analysis:* 11·41 % Cl.

A compound of the formula $C_{27}H_{47}Cl$ would require 8·76 %; $C_{24}H_{40}Cl$, 9·74 %; $C_{27}H_{46}Cl_2$, 16·6 %.

This oil on heating at 110° in an air bath gave off a pungent acid substance, probably hydrochloric acid. It was heated until it ceased to smell, taken up in light petroleum, filtered from a slight brown residue and the petroleum evaporated. The brown oil so obtained contained 6·05 % Cl. Another specimen treated in the same manner gave exactly the same result.

A specimen, B.P. 205–215°, which had been made from oil not absolutely freed from coprosterol by digitonin was treated in a similar manner. The product was reduced by sodium and alcohol in the hope of obtaining a hydrocarbon. For this purpose the product from 0·8 g. of material was dissolved in boiling alcohol in a flask under a reflux and 5 g. of sodium gradually added. After all the metal had dissolved, water was added. An oil separated but no trace of crystalline matter. This was taken up in ether and the solution thoroughly washed with water and dried. After evaporating the ether an oil was obtained of pretty much the same weight as the original material. It was difficultly soluble in hot alcohol, but readily in acetone, ether and chloroform.

Attempts to crystallise it were unsuccessful and it was therefore distilled at a pressure of about 1 mm. It came over on a metal bath heated to 225–240°, and was obtained as a yellow oil which set on cooling to a transparent glass. No residue remained in the flask.

On analysis it was found to contain 85·42 % C, 11·57 % H. The material, however, still contained traces of chlorine. The experiment was therefore repeated using 2·2 g. of a specimen from the bread subjects, B.P. 220–230°. The chloro-derivative was reduced with 14 g. of sodium. The product isolated still contained traces of chlorine. It was therefore dissolved in 100 cc. of amyl alcohol and again reduced with sodium at the boiling point. Water was then added and after standing over night the amyl alcohol was distilled off in steam. The residual oil was taken up in ether and benzene. It could not be got to crystallise and was therefore distilled in a vacuum of 2 mm. or thereabouts. It slowly evaporated over leaving no residue, the temperature indicated by the thermometer in the vapour being 200–214°.

At the laboratory temperature (16°) the distillate was a clear yellow very viscid oil which could just flow. It was free from chlorine and contained 83·74 % C, 11·89 % H, figures which are much the same as those required for cholesterol. Evidently a hydrocarbon had not been obtained. Possibly the chlorine might have been replaced by an ethoxy-group. A substance of the formula $C_{27}H_{45}O.C_2H_5$ would contain 84·06 % C, 12·12 % H.

On dissolving in chloroform and adding excess of acetic anhydride and a few drops of sulphuric acid a deep reddish brown colour was produced, which rapidly changed to sage green and finally bright green.

Some of the fractions on combustion showed a rather high carbon content, suggesting possibly the presence of a small amount of some hydrocarbon. Fraction (9) from the soldiers' faeces (p. 263) is a case in point. Attempts, however, to isolate any such substance in a crystalline form failed.

0·8 g. of this particular fraction was dissolved in ether and 5 cc. acetic anhydride and a few drops of sulphuric acid added. The usual green colour developed, which slowly faded to brown. After standing several days nothing of a solid nature separated. The whole was again dissolved in ether, 1 cc. of sulphuric acid added, and the solution left to stand for the ether to evaporate spontaneously. After standing many days no solid deposit separated and a thin black fluid remained. On dilution largely with water it gave a homogeneous solution. On addition of alkali, however, a sticky substance separated in flocks. This was filtered off, but on washing with water appeared to dissolve again to a colloidal solution. On again adding alkali the flocculent precipitate reappeared. This was taken up in ether, dried and on again evaporating the ether a stiff oil was obtained very similar in appearance to the original material.

Wacker [1912] stated that the portion of unsaponifiable matter of the fat of tissues not precipitated by digitonin was unstable to alkali, and when boiled with alcoholic potash gave a "resin" and a "fatty acid."

From the mode of preparation and treatment of these amorphous substances of the faeces it seemed unlikely that they would be affected by alcoholic potash, but it was possible that they might contain traces of stable esters that had perhaps escaped hydrolysis in the ethoxide saponification and subsequent treatment.

A number of the fractions were therefore separately heated in sealed tubes with excess of strong alcoholic soda for several hours at 110°. After cooling the contents of the tubes were diluted with water and extracted with ether.

The oils were in every case recovered quantitatively, and, judging by boiling point, general properties and combustion, apparently unchanged. On treatment with digitonin no weighable quantity of insoluble digitonide was obtained. Evidently therefore these clear transparent fractions were stable to alkalis, and contained no esters, though they may have contained minute traces of cholesterol or coprosterol.

*Origin of these amorphous substances.*

Two sources suggest themselves, (1) the substances which accompany cholesterol in the unsaponifiable matter of tissue fat, (2) the bile acids or their derivatives.

(1) The sterols precipitable by digitonin constitute only a portion of the unsaponifiable matter of the fat of tissues and organs. Wacker [1912] describes what he called "Begleitsubstanzen des Cholesterins" in human depot fat. He found that on an average the underskin fat contained 65·8 and the mesenterial fat 61·3 % of this substance.

He describes it as a wax-like body melting at 25–32°, which possesses properties analogous to those of cholesterol. It easily emulsifies with water. It is easily extracted from these emulsions by means of ether, otherwise the emulsions persist for a long time. It is soluble in most organic solvents—alcohol, ether, light petroleum, glacial acetic acid—but cannot be made to crystallise. He also states that this "Begleitsubstanz" gives in chloroform solution with acetic anhydride and sulphuric acid a brownish red coloration, but does not mention any further changes in colour.

It was unchanged by acetic anhydride and not precipitated by digitonin.

As mentioned above he states that this wax was unstable to alkalis, and if boiled with sodium ethoxide or alcoholic potash on the water bath there separated after dilution with water a brown resin. The alkaline filtrate on acidification gave "apparently a fatty acid." He does not give any indication of the quantity or nature of this acid.

This description we are able to confirm. A considerable quantity of human omental fat was hydrolysed and the unsaponifiable matter precipitated by digitonin. The oil separated by means of ether from the digitonin precipitate was in the cold of a waxy nature, and when treated in chloroform solution with acetic anhydride and sulphuric acid gave a brownish red colour, which

after a few minutes developed a greenish fluorescence. This became more marked on standing, but on long standing faded to brown.

On distillation in superheated steam it volatilised readily, condensing in the condenser in the white candles of solid emulsion described in the case of the faecal oils. Practically no residue remained either in the trap or in the distilling flasks.

Human muscle was also extracted and treated in a similar manner. The oil distilled in superheated steam in the same characteristic fashion.

Both the above distillates were taken up in ether, and the solutions dried and evaporated. In both cases the oil passed over in a high vacuum at 185–200° leaving only a trace of tarry matter.

A considerable amount of material has been accumulated from various sources, and we hoped to be able to purify this and compare it with the faecal wax. We have, however, been as yet unable to complete this work owing to difficulty in obtaining digitonin in sufficient quantity since the war. We hope, however, to investigate the material more fully later on.

In a series of papers published during the past ten years, J. Lipschütz [1908–1914] has put forward the view that in the general cholesterol metabolism the first oxidative attack on cholesterol takes place in the blood stream with the formation of an oxycholesterol. This substance he has also found in the unsaponifiable matter of the fat of other tissues. The oxycholesterol he estimates [1913, 1 and 2] by means of a colour reaction, with glacial acetic acid and sulphuric acid, not given by cholesterol itself. As in the case of the Burchardt-Liebermann reaction for cholesterol, this colour change is a progressive reaction, and the last stage is used. This usually takes some time to attain, but can be hastened by the addition of a little 5 % solution of iron chloride in glacial acetic acid [1908, 1, 2 and 3]. He does not compare the colours in an ordinary colorimeter, but uses a comparative spectroscope and compares the spectral intensities of the absorption bands of the chloroform solution of the substance under examination with those of a standard solution of "pure" oxycholesterol, the stronger solution being diluted until equality of absorption is attained. The oxycholesterol for his test solutions he makes by the action of benzoyl superoxide on cholesterol. It is a resinous substance, showing no tendency to crystallise. It dissolves in all media except water, but particularly easily in benzene and methyl alcohol. It has no sharp melting point and has no ordinary property which can serve as a criterion of purity. Lipschütz uses the spectral intensity of the bands in the above-mentioned colour reaction for this purpose.

In order to compare this substance with the faecal distillates it was prepared following exactly Lipschütz's instructions [1914]. The various phenomena noticed and the final product agreed exactly with the descriptions given in his paper. He states that oxycholesterol, free from cholesterol, is precipitated from its solutions by digitonin, similarly to cholesterol, in small white silvery crystals melting with decomposition at 218°. The weight obtained corre-

sponded to 50–53 % of the oxycholesterol used. Most people would, we think, have interpreted this result as indicating that the oxycholesterol used was not a pure substance, but contained 50 % of some sterol precipitable by digitonin. Lipschütz, apparently on account of the spectral properties, does not draw this conclusion, but regards his substance as being only partially precipitable. In these experiments he followed the procedure described by Windaus [1910] for the estimation of cholesterol. His conclusion would only be understandable if the reaction were a reversible one, or if the oxycholesterol-digitonide were to some extent soluble in alcohol. In either case we should expect the percentage of substance precipitated to vary with the conditions of reaction.

In order to test this point the precipitable "oxycholesterol" was estimated by the modified procedure described by Fraser and Gardner [1910] in which the whole of the alcoholic solvent is evaporated before washing away oil and excess of digitonin. In this way it was found that 49·4 % of the material was precipitated, a result practically the same as that given by Lipschütz. The portion not precipitated was washed away from the compound and excess of digitonin by means of ether. In chloroform solution this gave with acetic anhydride and sulphuric acid a reddish brown colour, which did not however change to green.

To compare the oxycholesterol with the faecal waxes it was distilled in superheated steam. A small portion passed over in the form of a white solid emulsion very similar in appearance to that obtained with other sterols, followed by a viscid yellow oil in somewhat larger quantity. Some decomposition appeared to have taken place, as a considerable amount of a non-volatile brown residue remained in the distillation flask.

The emulsion and the oil were separately taken up in ether and the solutions were dried and evaporated. The oil from the emulsion was found to contain 18·5 % of substance precipitable by digitonin, reckoning it as cholesterol. The part which was not precipitated was dissolved out by ether, and in chloroform solution gave with acetic anhydride and sulphuric acid a brownish red colour which in a few minutes changed to a dusky green. It gave no colour when glacial acetic acid was substituted for acetic anhydride, even on long standing.

The second portion of the distillate, which dried to a sticky mass, was found to contain 25·36 % of a sterol precipitable by digitonin. In chloroform solution it gave with acetic anhydride and sulphuric acid a pinkish brown colour which rapidly changed through deep blue to green, a series of changes much more like those given by cholesterol and coprosterol. The oil washed away from the digitonide gave a reddish brown colour which rapidly turned green, but without any trace of a blue stage. On standing the green gradually faded and left a dirty brown fluid. With glacial acetic acid instead of anhydride it gave a reddish colour, which did not change.

Lipschütz's product, whether a single substance or not, was quite different from the amorphous substance from faeces.

(2) Another source that suggests itself for the sterol waxes of the faeces is the bile acids.

It has long been suspected that cholic acid, the most specific bile acid, and the sterols are members of a hydroaromatic class of polycyclic type, and probably possess a close chemical and genetic relation to one another. It is well known that cholesterol is normally eliminated by the liver, and in 1913 Grigaut stated as his own personal opinion that only a small part of the cholesterol of the cells dealt with by the liver is excreted as cholesterol, the major portion appearing in the bile in the form of a product of transformation —cholic acid, so that according to him cholesterol plays an important part in the formation of bile salts and cholic acid represents in the bile the principal product in the elimination of the cholesterol of the organism. It must be admitted however that he produces little convincing evidence for this view.

Cholic acid and its derivatives, however, give rise to colour reactions quite similar to those of cholesterol. Thus Wieland and Weil [1912] found that cholatrienic acid prepared from cholic acid gave the Burchardt-Liebermann test, and Lipschütz a little later pointed out that cholesterol and cholic acid, when boiled with glacial acetic acid, benzoyl superoxide and concentrated sulphuric acid, gave similar highly characteristic colour reactions.

Clear proof of the connection, however, follows from the recent work of Wieland and his co-workers on bile acids and of Windaus and his co-workers on cholesterol.

In 1912 Wieland and Weil obtained, by the distillation of cholic acid *in vacuo*, an unsaturated acid, cholatriencarboxylic acid, $C_{24}H_{34}O_2$. This gave on catalytic reduction by hydrogen in presence of palladium a less unsaturated acid, $C_{24}H_{36}O_2$, and finally a saturated monobasic acid—cholanic acid, $C_{24}H_{40}O_2$.

In 1919 this same cholanic acid, $C_{24}H_{40}O_2$, was prepared by Windaus and Neukirschen [1920] by oxidation in glacial acetic acid by chromic acid of $\psi$-cholestan, so that cholic acid and $\psi$-cholestan, a derivative of coprosterol, must have the same carbon skeleton.

In order to obtain, if possible, some indication as to whether the oils of faeces, boiling above 200°, belonged to the sterol group with 27 carbon atoms, or to the bile acid group with 24, they were submitted to drastic oxidation, and we may briefly mention the preliminary results.

2·5 g. of a fraction 200–211° were dissolved in glacial acetic acid on the water bath and 10 g. of chromic acid gradually added. After heating for several hours the mass was slowly distilled. The distillate was made alkaline and again distilled. This distillate contained some acetone which was recognised by the iodiform test and Marsh's test. The residue in the flask after dilution was extracted with ether, and the neutral and acid products separated in the usual way. The acid was a stiff oil which would not crystallise, but on titration was found to have an average molecular weight of 384. The neutral product weighed 1·5 g. and appeared to be unchanged material.

In another experiment 1·4 g. similarly treated gave 0·019 g. of acetone,

estimated by Scott-Wilson's method [1911]. On evaporating the neutral ether solution the fragrant smell often given in sterol oxidations was noticed. The quantity was much too small for any attempt at identification, but it was probably due to the methyl*iso*hexyl ketone isolated by Windaus and Resau [1913]. In another experiment the oil was submitted to drastic oxidation by means of fuming nitric acid. It was hoped that the results might give some indication as to whether the wax originated from a sterol of the cholesterol group or from bile derivatives. It has been shown by Bredt that dinitro*iso*propane is usually formed by the oxidation of substances containing an isopropyl grouping with nitric acid. Windaus [1918] isolated this substance by the energetic oxidation of cholesterol with strong nitric acid. It is not, however, formed when the $C_{24}$ acid—cholic acid—is oxidised in a similar manner, as this does not possess an *iso*propyl-group. The three carbon atoms constituting the difference between the carbon skeletons of cholesterol and cholic acid are apparently in the form of such a grouping.

12 g. of wax, 200–202°, were oxidised with 300 cc. of fuming nitric acid in a very long necked flask. The reaction was very vigorous at first and started at the ordinary temperature, quantities of nitrous fumes being evolved.

As soon as the action slackened, the flask was put on the water bath. Finally the liquid was boiled over a gauze for several hours until the evolution of fumes had nearly ceased. The product was then dealt with in the manner described by Windaus [1918]. In order to isolate the volatile products half the solution was distilled off. The distillate was neutralised with caustic soda and again submitted to distillation. In the distillate very small quantities of oily drops were noted, apparently insoluble in water. They appeared to solidify on cooling and had a characteristic smell, but the quantity was much too small for any attempt at identification. The smell was quite consistent with this being dinitro*iso*propane, but it could not be further identified. In any case the quantity was very minute. The neutral fluid in the distilling flask was then acidified with sulphuric acid until distinctly acid to tropaeolin paper, and again distilled. The strongly acid distillate was boiled with silver carbonate, when a considerable amount of silver separated. This was filtered off and the filtrate evaporated *in vacuo* over sulphuric acid. A considerable quantity, over 1 g., of silver acetate was obtained in needle shaped crystals.

On analysis this gave (1) 63·9 % Ag; (2) 62·44 % Ag; (3) 63·76 % Ag; calculated for silver acetate, 64·65 % Ag.

The main quantity of volatile acid was evidently acetic acid.

The nitric acid liquor, left after separating the volatile acids, was evaporated in a platinum dish with frequent addition of water to get rid of nitric acid. The residue was a brown syrup which showed no sign of crystallising on standing. This was dissolved in water and neutralised with milk of lime, when a white precipitate containing a good deal of calcium oxalate was formed. This was filtered and the filtrate precipitated with lead acetate. The bulky, heavy precipitate formed was filtered off. The acid obtained from this salt was

amorphous. It gave a soluble barium salt containing 39·2 % of barium. It was not further examined, but was perhaps similar to the amorphous acid obtained by Mauthner and Suida [1903] by oxidising cholesterol with nitric acid.

After separating the excess of lead from the filtrate from the lead salt by means of sulphuric acid, the liquid was evaporated to small bulk and extracted thoroughly with ether. After evaporating the ether the extract was placed in a small retort and heated in an air bath to 220°. It was then cooled and slowly distilled *in vacuo*. At first an aqueous distillate was obtained and at a higher temperature an oil passed over which solidified in the receiver. The distillate was dissolved in hot water and treated with baryta in slight excess and boiled. There separated a small quantity of a difficultly soluble barium salt. This was quantitatively decomposed by dilute sulphuric acid, and after filtering off the barium sulphate the filtrate was evaporated to dryness and boiled out with benzene. The insoluble acid was then recrystallised from water. It melted at 185° and was identified as succinic acid. There was not, however, sufficient for a combustion. The filtrate from the barium succinate was acidified with hydrochloric acid and extracted with ether. After evaporating the ether the residue was completely soluble in benzene, and evidently contained no more succinic acid.

The benzene solution was mixed with light petroleum and on standing crystals were deposited. These were recrystallised from these solvents several times and finally from water. The melting point was now constant—149–150°.

The equivalent weight (by titration) was 71·3.

*Analysis:* 47·42 % C, 7·57 % H; calculated for $C_6H_{10}O_4$, 49·31 % C, 6·85 % H. The acid was probably adipic acid.

A very small quantity of another acid was obtained from the benzene mother liquors, melting at 118°. On repeated recrystallisation from water the melting point was raised to 127–128°. Titration indicated an equivalent of 80, or if dibasic, a molecular weight of 160. This would fit an acid of the formula $C_7H_{12}O_4$.

The mother liquors on evaporation gave an oil which on standing solidified to a wax. Titration gave an equivalent of 83·4. After recrystallisation from water it shrank together at 94° and melted at 101–102°. It was not further examined.

These preliminary experiments were inconclusive as far as the settlement of the alternative origin suggested is concerned.

The oxidation of this substance is, however, being studied in greater detail, and feeding experiments are in progress which it is hoped will throw light both on the constitution and origin.

The writer feels some diffidence in bringing forward so long an account of a substance, obviously not pure, and perhaps a mixture, but his justification for doing so is that whatever its exact nature it undoubtedly belongs to the sterol or polyterpene group, and in any metabolic experiments dealing with the intake and output of sterols must be taken into account.

In conclusion the writer wishes to express his thanks to Mr F. W. Fox for his help in most of the analytical work in this investigation, and to the Government Grant Committee of the Royal Society for help in carrying out this work.

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# XXXI. THE ANTISCORBUTIC AND ANTIBERI-BERI PROPERTIES OF CERTAIN SUN-DRIED VEGETABLES.

By JAMES ALFRED SHORTEN and CHARU BRATA RAY.

*From the Physiological Laboratory, Medical College, Calcutta, India.*

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The present investigation is a continuation of work undertaken at the request of Sir Leonard Rogers to determine the antiscorbutic and antiberi-beri properties of certain sun-dried vegetables prepared at the Government Fruit Experiment Station, Quetta [Shorten and Ray, 1919]. This paper dealt with four varieties of vegetables, viz. carrots, spinach, onions, and cabbage. The period of experimental feeding—42 days—was afterwards considered too short. There were also certain accidental irregularities in the feeding which made it desirable to repeat the experiments.

As stated in our original paper [1919], the specimens of vegetables experimented with were prepared under the direction of Mrs G. L. C. Howard, Second Imperial Economic Botanist to the Government of India. A full description of the methods employed is to be found in Bulletin No. 8 of the Fruit Experiment Station, Quetta, dated March 1918. A short description of the procedure adopted may here be quoted from our original paper.

"The vegetables having been cleaned and prepared are placed in fresh water, or in certain cases in a steeping solution. The prepared material is then transferred to a wire basket and either scalded or steamed for a few minutes. The contents of the wire basket are now emptied on to the drying trays and dried in the sun. The dried product is collected in sacks or open boxes in which it is allowed to remain for about a week, during which time it is examined at intervals for signs of dampness. After about a week the dried product is ready to be compressed into bricks and packed in airtight tins."

The present investigation comprises experiments with nine varieties of vegetable, viz. carrots, brinjal, spinach, cabbage, tomatoes, potatoes, turnips, turnip-tops and mixed factory-dried vegetables prepared by Messrs Swallow and Ariel, Port Melbourne, Australia.

In the case of the sun-dried vegetables the samples were from three to six months old. The age of the mixed factory-dried product could not be ascertained. All the samples were in good condition when opened. On opening the tins they were made up in 5 g. packets and stored at room temperature over sulphuric acid, beneath glass bell-jars. This precaution was necessary to prevent

deterioration in the damp climate of Calcutta. The daily temperature varied between 80° and 90° Fahrenheit. The time during which the vegetables were thus stored before consumption did not exceed two weeks in most cases.

Preliminary experiments showed that guinea-pigs readily consumed, and throve on, 50 g. of fresh vegetable daily. Holst and Frölich [1912], and later Delf [1918], had previously shown that guinea-pigs remain scurvy free and put on weight on 30 g. of fresh cabbage daily in addition to a scurvy producing basal diet. In our experiments we thought it advisable to use a larger daily ration to allow for the less nourishing properties of vegetables grown in India, and for possible variations in the vitamin content of different varieties of fresh vegetable.

In the case of sun-dried vegetables it was found that 1 g. dried vegetable corresponds roughly to 10 g. fresh vegetable. The ration of dried vegetable chosen was therefore 5 g. and it was found that this amount was readily consumed in most instances.

Six guinea-pigs were used for each experiment, all the animals were placed on a basal diet consisting of crushed oats, bran, hay and water *ad libitum*, to which was added daily 30 cc. of whole milk autoclaved at 120° C. for one hour. Two animals were given 50 g. fresh vegetable daily; two 5 g. of the sun-dried product soaked in cold water; and two 5 g. of sun-dried product cooked for about 30 mins., according to the directions given by the producers (boiled in water and the liquor strained off). The animals were kept in open cages and were allowed to run about for a short time daily. They were weighed every fourth day under the supervision of one of us, and carefully inspected for clinical signs of scurvy, when the onset of the symptoms was expected. Post mortem examinations were carried out on all animals that died and histological examinations of the rib-junctions and other parts when necessary.

To determine the antineuritic properties, common fowls, weighing between 1200 and 2000 g., were used. These birds were given a basal diet of Rangoon polished rice with water *ad libitum*, to which was added 10 g. of the sun-dried product under investigation. Regular weighings and inspections were carried out as in the case of the guinea-pigs.

*Sun-dried carrots* (Exp. 1, Fig. 1).

A. Control; guinea-pigs Nos. 1 and 2—basal diet with 50 g. fresh carrots daily.

*Remarks:* Both animals remained healthy for 91 days. No. 1 gained 75 g. in weight, and No. 2 10 g.

B. Guinea-pigs Nos. 13 and 14—basal diet with 5 g. sun-dried carrots soaked in water, but not cooked.

*Post mortem report:* Both animals showed typical signs of scurvy—enlargement of the costo-chondral junctions with haemorrhages, destruction of the cartilage columns at the junctions, and haemorrhages into various joints.

In No. 1 there was a subserous haemorrhage in the bladder. No. 2 had haemorrhages in the kidneys. All other organs were healthy.

*Remarks:* No. 1 lived 36 days and No. 2, 30 days, indicating a slight degree of protection as compared with animals on the basal diet only. Both animals developed diarrhoea a few days before death, which probably hastened the fatal issue.

C. Guinea-pigs Nos. 25 and 26—basal diet with 5 g. sun-dried carrots cooked as directed.

*Post mortem report:* No. 25 showed typical signs of scurvy, enlargement of the costo-chondral junctions with haemorrhages, subserous haemorrhages in the bladder. Subcutaneous and deep haemorrhages in the knee-joints. Other organs healthy.

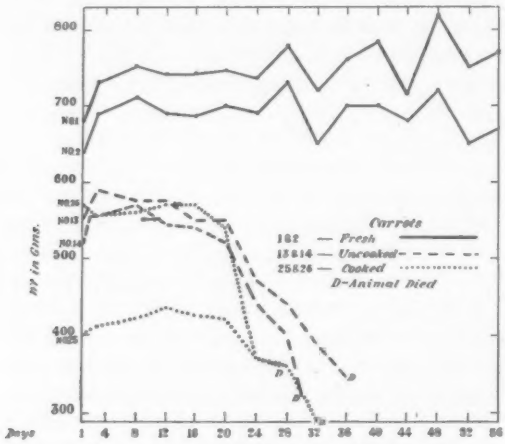


Fig. 1. Experiment No. 1. Sun-dried carrots.

No. 26 had a haemorrhage in the broad ligament but no other signs of scurvy.

*Remarks:* No. 25 lived 32 days showing no protection as compared with controls on the basal diet.

The death of No. 26 was probably hastened by the fact that it aborted on the 21st day, giving birth to two dead foetuses. It died on the 27th day before the symptoms of scurvy were fully developed.

*Sun-dried brinjal* (Exp. 2, Fig. 2).

A. Control. Guinea-pigs Nos. 3 and 4—basal diet with 50 g. fresh brinjal daily.

*Remarks:* Healthy for 91 days. No. 3 gained 50 g., and No. 4 130 g.

B. Guinea-pigs Nos. 15 and 16—basal diet with 5 g. sun-dried brinjal soaked in water, but not cooked.

*Post mortem report:* Both animals showed typical signs of scurvy. In the case of No. 15 all the costo-chondral junctions were enlarged and haemorrhagic. There were haemorrhages into and around the knee-joints. No. 16 showed enlargement of and haemorrhage into the costo-chondral junctions. Microscopical examination of sections showed destruction of the cartilage columns, and haemorrhages at the costo-chondral junctions.

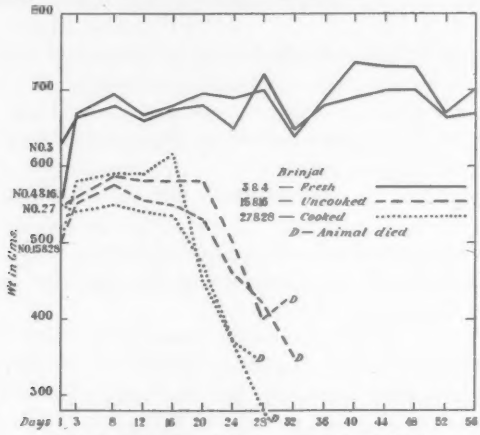


Fig. 2. Experiment No. 2. Sun-dried brinjal.

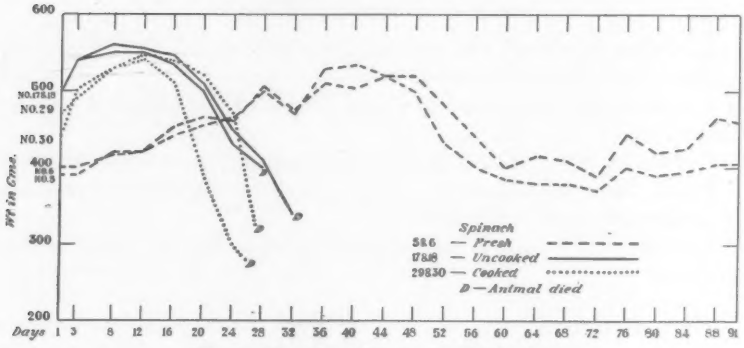


Fig. 3. Experiment No. 3. Sun-dried spinach.

*Remarks:* No. 15 lived 32 days and No. 16, 31 days, thus showing no protection as compared with the control animals.

C. Guinea-pigs Nos. 27 and 28—basal diet with 5 g. cooked sun-dried brinjal.

*Post mortem report:* Both animals exhibited typical signs of scurvy. No. 27 had haemorrhages in the bladder and broad ligament.

*Remarks:* No. 27 lived only 27 days; No. 28, 29 days, thus showing no evidence of protection.

*Sun-dried spinach* (Exp. 3, Fig. 3).

A. Control. Guinea-pigs Nos. 5 and 6—basal diet with 50 g. fresh spinach daily.

*Remarks:* Healthy throughout, but the gain in weight was not as marked as in some of the other groups.

B. Guinea-pigs Nos. 17 and 18—basal diet with 5 g. of soaked sun-dried spinach daily.

*Post mortem report:* Both animals showed marked signs of scurvy—enlarged and haemorrhagic costo-chondral junctions; haemorrhages into joints and various organs, subcutaneous and intra-muscular haemorrhages.

*Remarks:* No. 17 lived 32 days, and No. 18, 28 days. Both animals developed diarrhoea as a terminal symptom.

C. Guinea-pigs Nos. 29 and 30—basal diet with 5 g. cooked sun-dried spinach.

*Post mortem report:* Both animals had typical scorbutic lesions in the costo-chondral junctions. No. 30 had a haemorrhage into the broad ligament. There were scybalae in the large intestine.

*Remarks:* It is remarkable that both animals lived a shorter time than the controls on basal diet only. No. 29 died on the 28th day, and No. 30 on the 29th day. This may have been due to the fact that both animals were pregnant. No. 29 aborted on the 18th day, and No. 30 was delivered of a dead foetus on the 26th day.

*Sun-dried cabbage* (Exp. 4, Fig. 4).

A. Guinea-pigs Nos. 7 and 8—basal diet with 50 g. of fresh cabbage daily.

*Remarks:* Healthy throughout.

B. Guinea-pigs Nos. 19 and 20—basal diet with 5 g. of uncooked sun-dried cabbage.

*Post mortem report:* No. 19 (killed with chloroform), no evidence of scurvy.

No. 20. There was some slight enlargement of the costo-chondral junctions, but no haemorrhages were found.

*Remarks:* No. 19 lived 91 days without any signs, clinical or post mortem, of scurvy.

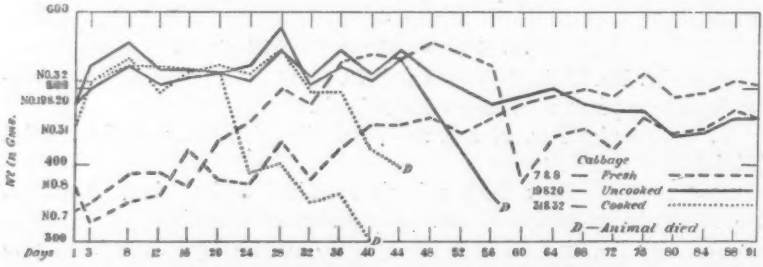


Fig. 4. Experiment No. 4. Sun-dried cabbage.

No. 20 maintained its weight for 48 days and then entered on a rapid decline dying on the 57th day. This animal also suffered from diarrhoea. The cause of death is unknown, careful macroscopic and microscopic examination revealed no definite signs of scurvy.

C. Guinea-pigs Nos. 31 and 32—basal diet with 5 g. sun-dried cabbage cooked as directed.

*Post mortem report:* No. 31, slight enlargement of the costo-chondral junctions—no haemorrhages.

No. 32, typical signs of scurvy in the costo-chondral junctions. Haemorrhages over the scapula. Organs healthy.

*Remarks:* The degree of protection shown here is less than in the case with cabbage fed after simple soaking in water. Animal No. 31 was apparently healthy till the 36th day, when it developed diarrhoea. It showed practically no signs of scurvy after death on the 44th day.

No. 32 aborted on the 23rd day and from that date declined rapidly, dying on the 40th day.

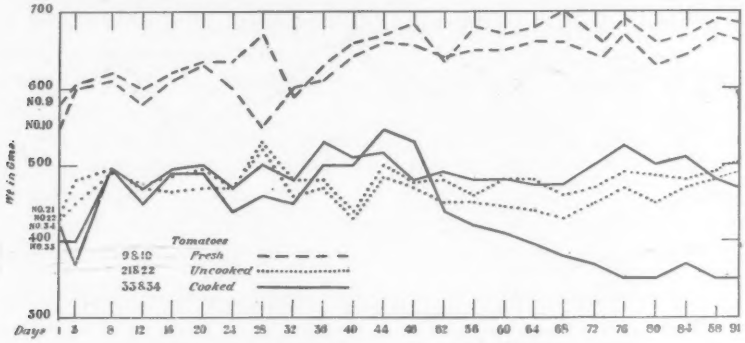


Fig. 5. Experiment No. 5. Sun-dried tomatoes.

*Sun-dried tomatoes* (Exp. 5, Fig. 5).

A. Guinea-pigs Nos. 9 and 10—basal diet with 50 g. fresh tomatoes daily.

*Remarks:* Healthy throughout. No. 9 gained in weight 105 g.; No. 10, 110 g.

B. Guinea-pigs Nos. 21 and 22—basal diet with 5 g. of soaked sun-dried tomatoes daily.

*Post mortem report* (on No. 21 killed with chloroform). No signs of scurvy.

*Remarks:* Both guinea-pigs appeared to be perfectly healthy when the experimental feeding was discontinued. No. 21 gained 65 g. and No. 22 60 g. in weight. Protection against scurvy would appear to be complete.

C. Guinea-pigs Nos. 33 and 34—basal diet with 5 g. sun-dried tomatoes cooked as directed.

*Post mortem report* (on No. 33 killed with chloroform). No evidence of scurvy.

*Remarks:* Both animals were healthy when the experiment was stopped. No. 33 suffered from diarrhoea from the 52nd to the 55th day, but recovered without special treatment. This fact may explain its loss in weight—50 g. No. 34 gained 50 g. in weight.

*Sun-dried potatoes* (Exp. 6, Fig. 6).

A. Guinea-pigs Nos. 11 and 12—basal diet with 50 g. fresh potatoes daily.

*Remarks:* This vegetable was not relished to the same extent as the preceding kinds. The average quantity consumed by both animals was about 20 g. daily. Both were healthy at the conclusion of the experimental period. No. 11 gained 35 g. in weight; and No. 12, 65 g.

B. Guinea-pigs Nos. 23 and 24—basal diet with 5 g. of soaked sun-dried potatoes daily.

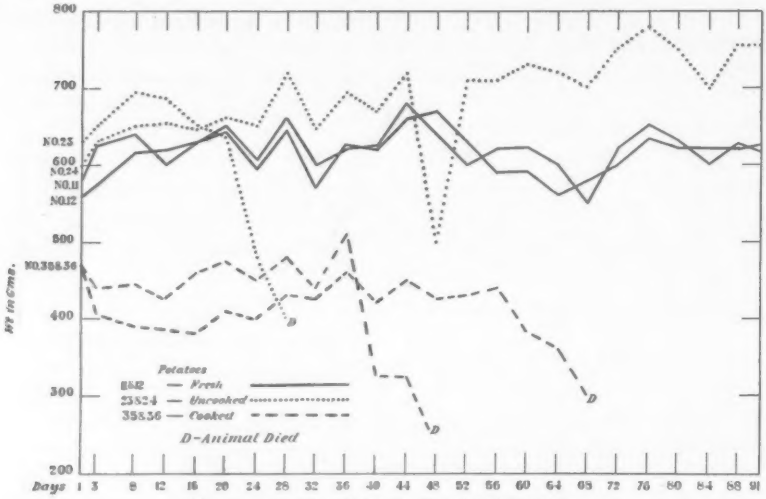


Fig. 6. Experiment No. 6. Sun-dried potatoes.

*Post mortem report:* On No. 23. No evidence of scurvy, costo-chondral junctions normal. No haemorrhages. Organs all appear healthy. Cause of death unknown.

*Remarks:* No. 23 appeared to be perfectly well till about the 20th day, when it began to lose weight. There was no diarrhoea. The animal refused to take food and died on the 28th day. There were no clinical manifestations of scurvy at any time. No. 24 continued to do well and had gained 155 g. in weight at the conclusion of the experiment.

C. Guinea-pigs Nos. 35 and 36—basal diet with 5 g. sun-dried potatoes cooked as directed.

*Post mortem report:* No. 35, no signs of scurvy, cause of death unknown.

No. 36. Enlargement of costo-chondral junctions with haemorrhages. Haemorrhage into bladder, subcutaneous and intra-muscular haemorrhages around the knee-joints.

*Remarks:* The soaked product would appear to afford almost complete protection against scurvy, the boiled product only a moderate degree.

*Mixed factory-dried vegetables* (Exp. 7).

A. Guinea-pigs Nos. 41 and 42—basal diet with 5 g. daily of mixed factory-dried vegetables soaked in water.

*Initial weights:* No. 41, 460 g.; No. 42, 415 g. *Final weights:* No. 41, 270 g.; No. 42, 320 g.

*Post mortem report:* Both animals showed the usual typical signs of scurvy —enlarged and haemorrhagic costo-chondral junctions, haemorrhages into joints and various organs.

*Remarks:* No. 41 lived 29 days; No. 42, 37 days. Both showed marked symptoms of scurvy before death.

B. Guinea-pigs Nos. 43 and 44—basal diet with 5 g. daily of mixed factory-dried vegetables cooked as usual.

*Initial weights:* No. 43, 580 g.; No. 44, 570 g. *Final weights:* No. 43, 350 g.; No. 44, 250 g.

*Post mortem report:* Both animals, more particularly No. 43, had enlarged and haemorrhagic costo-chondral junctions, subcutaneous and joint haemorrhages.

*Remarks:* No. 43 lived 26 days; No. 44, 23 days. Both had marked clinical signs of scurvy before death.

*Sun-dried turnip-tops* (Exp. 8).

A. Guinea-pigs Nos. 45 and 46—basal diet with 5 g. soaked sun-dried turnip-tops daily.

*Initial weights:* No. 45, 350 g.; No. 46, 370 g. *Final weights:* No. 45, 305 g.; No. 46, 290 g.

*Post mortem report:* Both animals had typical signs of scurvy—enlarged and haemorrhagic costo-chondral junctions, haemorrhages into joints and other parts.

*Remarks:* Both animals lived 47 days and exhibited well-marked clinical signs of scurvy before death. No. 45 developed diarrhoea on the 38th day.

B. Guinea-pigs Nos. 47 and 48—basal diet with 5 g. of boiled sun-dried turnip-tops daily.

*Initial weights:* No. 47, 520 g.; No. 48, 480 g. *Final weights:* No. 47, 310 g.; No. 48, 310 g.

*Post mortem report:* No. 47 had enlargement of the costo-chondral junctions with extensive haemorrhages into knee-joints, muscles, etc. No. 48 had enlargement of the costo-chondral junctions, but no haemorrhages.

*Remarks:* No. 47 lived 44 days and showed well-marked clinical symptoms of scurvy before death. No. 48 did not have any definite symptoms of scurvy, when it died on the 47th day.

*Sun-dried turnips* (Exp. 9).

A. Guinea-pigs Nos. 49 and 50—basal diet with 5 g. uncooked sun-dried turnips daily.

*Initial weights:* No. 49, 570 g.; No. 50, 520 g. *Final weights:* No. 49, 355 g.; No. 50, 400 g.

*Post mortem report:* There was some enlargement of the costo-chondral junctions in both cases. Haemorrhages were not marked.

*Remarks:* No. 49 lived 32 days and No. 50, 37 days; neither showed very marked clinical or other evidence of scurvy.

B. Guinea-pigs Nos. 51 and 52—basal diet with 5 g. of boiled sun-dried turnips daily.

*Initial weights:* No. 51, 400 g.; No. 52, 450 g. *Final weights:* No. 51, 225 g.; No. 52, 250 g.

*Post mortem report:* Both animals, especially No. 51, showed typical signs of scurvy.

*Remarks:* Practically no protection.

*Control; basal diet only* (Exp. 10).

Guinea-pigs Nos. 37 and 38.

*Initial weights:* No. 37, 530 g.; No. 38, 520 g. *Final weights:* No. 37, 350 g.; No. 38, 390 g.

*Post mortem report:* Both animals exhibited the typical post mortem signs of scurvy. The costo-chondral junctions were enlarged and haemorrhagic, and under the microscope showed destruction of the cartilage columns. There were extensive subcutaneous haemorrhages in both legs, haemorrhages into and around the knee-joints. No. 38 had submucous haemorrhages in the bladder. The teeth were loose and the gums congested. The jaw bone was brittle and broke easily. No. 37 had no marked changes in the teeth and gums.

*Remarks:* Before death both animals exhibited the typical symptoms of scurvy—lameness, face ache position, etc. Signs of scurvy first appeared about the 25th day and both animals died on the 32nd day.

*Antineuritic properties* (Exp. 11, Fig. 7).

Fowl No. 1. Basal diet—Rangoon rice with 10 g. boiled sun-dried carrots daily (see Fig. 7).

*Remarks:* At the conclusion of the experiment the bird was quite healthy and had gained 180 g. in weight.

Fowl No. 2. Basal diet with 10 g. boiled sun-dried brinjal daily (see Fig. 7).

*Remarks:* The bird, though it had lost 290 g. in weight, was apparently healthy and showed no signs of neuritis.

Fowl No. 3. Basal diet with 10 g. of boiled sun-dried spinach daily (see Fig. 7).

*Remarks:* This bird remained healthy and gained 120 g. in weight.

Fowl No. 4. Basal diet with 10 g. of boiled sun-dried cabbage daily (see Fig. 7).

*Remarks:* This bird gained 430 g. in weight and was healthy and vigorous at the end.

Fowl No. 5. Basal diet with 10 g. of boiled sun-dried tomatoes daily (see Fig. 7).

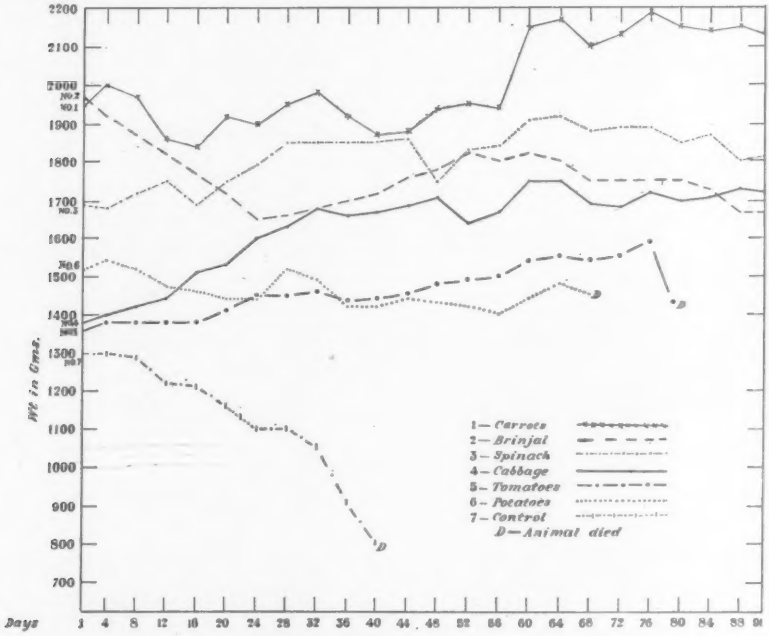


Fig. 7. Antineuritic properties.

*Post mortem report:* Organs healthy, cause of death could not be ascertained. On histological examination the sciatic nerves were found to be normal and there was no sign of any degeneration.

*Remarks:* The bird was quite healthy until it suddenly died on the 79th day, having gained 70 g. in weight. There were no clinical signs of neuritis.

Fowl No. 6. Basal diet with 10 g. of boiled sun-dried potatoes daily (see Fig. 7).

*Post mortem report:* Organs healthy. On histological examination no signs of nerve degeneration could be found.

*Remarks:* This bird lost 70 g. in weight, otherwise it was quite healthy until the 68th day when it suddenly died—clinically there was no sign of neuritis.

Fowl No. 7. Control—basal diet only (see Fig. 7).

*Post mortem report:* Much emaciated. The skin of the legs was dry and scaly. No appreciable swelling was noticed. Macroscopically the organs were normal. On histological examination the sciatic nerves showed extensive degeneration—the axis cylinders of practically all the nerves had broken up, and the medullary sheath was converted into myelin droplets.

*Remarks:* On the 16th day the bird began to look unhealthy and gradually developed neuritis with its clinical symptoms. On the 33rd day he could scarcely stand, and moved with high stepping gait. He lost 500 g. in weight and died on the 40th day.

## Discussion.

From the short description of the process by which sun-dried products are prepared, quoted from our original paper, it will be noted that the drying process is carried out at the ordinary atmospheric temperature at Quetta, which varies between 75° and 100° F. during the drying season. They would therefore correspond in the main to the low-dried products of Givens and Cohen [1918] and Givens and McClugage [1919]. To what extent the preliminary scalding or steaming affects the process can only be determined by further experiments which we propose to carry out shortly. It is probable however that, in the case of thick slices, only the superficial cells are raised to a high temperature, and the central cells may to some extent escape the effect. The structure of the vegetable cells themselves may also have an influence, the coarser thick-walled types being more resistant.

From Table I we see that sun-dried tomatoes afford complete protection when fed both uncooked and cooked. Cabbage and potatoes afford partial protection, the degree of protection being more marked with the uncooked material. Tomatoes and potatoes are cut in comparatively thick slices and cabbage has a somewhat coarse leaf. The texture of brinjal and spinach, two varieties which show absolutely no antiscorbutic properties, is comparatively delicate.

A point which appears to emerge from Table I is the influence of cooking. Animals on the uncooked product lived longer in almost every instance than those on a similar ration fed in the cooked state.

Table I. *Antiscorbutic properties.*

| Vegetable | No. of days guinea-pigs lived on 5 g. daily in addition to basal diet | |
|---|---|---|
| | Uncooked | Cooked |
| Sun-dried carrots ... | 36, 30 | 32, 24 |
| ,, brinjal ... | 33, 31 | 27, 29 |
| ,, spinach ... | 32, 28 | 26, 27 |
| ,, cabbage ... | 91, 57 | 40, 44 |
| ,, tomatoes ... | 91, 91 | 91, 91 |
| ,, potatoes ... | 26, 91 | 37, 47 |
| ,, turnips ... | 37, 32 | 39, 35 |
| ,, turnip-tops ... | 50, 50 | 46, 43 |
| Mixed factory-dried ... | 32, 23 | — |
| Control ... ... ... | 32, 32 | — |

Table II. *Antineuritic properties.*

| Vegetable | No. of days fowls lived on 10 g. daily in addition to basal diet | Remarks |
|---|---|---|
| Sun-dried carrots ... | 91 | No evidence of neuritis |
| „ brinjal ... | 91 | „ „ |
| „ spinach ... | 91 | „ „ |
| „ cabbage ... | 91 | „ „ |
| „ tomatoes ... | 79 | No evidence of neuritis. Cause of death unknown |
| „ potatoes ... | 68 | „ „ |


## Summary.

1. Nine varieties of Quetta sun-dried vegetables have been investigated as regards their antineuritic and antiscorbutic properties.

2. Sun-dried tomatoes, potatoes and cabbage retain to a considerable extent the antiscorbutic properties of the fresh vegetable, when fed as a daily ration of 5 g. dried product to guinea-pigs weighing on an average 500 g.

3. Sun-dried carrots, brinjal, spinach, turnips and turnip-tops have little or no antiscorbutic properties.

4. Mixed factory-dried vegetables are entirely devoid of antiscorbutic properties.

5. Cooking before consumption diminishes any antiscorbutic vitamin which may be present. These products should therefore be given in the form of salads when possible.

6. Six varieties of Quetta sun-dried vegetables, viz. carrots, brinjal, spinach, cabbage, tomatoes and potatoes, were investigated as regards their antineuritic properties. In no instance was there any evidence of neuritis in common fowls weighing on the average 1600 g. on a daily ration of 10 g.


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# XXXII. ON AN AUTOXIDISABLE CONSTITUENT OF THE CELL.

By FREDERICK GOWLAND HOPKINS.

*From the Biochemical Laboratory, Cambridge.*

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## Section I. Introductory.

The research described in this paper, though its actual point of departure had an intention quite different[1], ultimately resolved itself into an endeavour to throw light upon the chemical nature and physiological significance of a constituent of living tissues, which, though hitherto of unknown nature, has long carried a name. There can scarcely be a doubt that the substance to be described is the "Philothion" of de Rey-Pailhade.

In 1888 this author showed that yeast cells and aqueous extracts of yeast have the property of reducing sulphur to hydrogen sulphide [de Rey-Pailhade, 1888]. Later he showed that many animal tissues possess the same property. Throughout a long series of communications upon the subject he has courageously maintained the view that the labile hydrogen thus shown to exist in living cells has important respiratory functions. His views as to the probable nature of the hypothetical substance (named as above) which he supposed to carry this labile hydrogen, have been modified from time to time. In his latest writings he speaks of it as the hydride of a protein ("hydrure d'albumine"). After the publication of Heffter, to which reference will immediately be made, he accepted the view that the labile hydrogen exists in sulphydryl groups, HS—.

In 1901 Mörner employed the delicate nitroprusside reaction for the identification of cysteïn, and a little later Gola found by its use that a substance, which presumably contains the HS— group, is characteristically present

[1] My attention was directed to the subject in two separate ways Some years ago I was endeavouring to discover if vitamins were to be found among sulphur-containing compounds, and was led part of the way towards the separation of the substance now described. A little later, acting on the suggestion that acid formation in muscle is a necessary factor in contraction I wished to discover if by chance in the absence of carbohydrate aceto-acetic acid from fat might function instead of lactic acid. This led me to apply the nitroprusside test to tissues. At this time Arnold's papers had not yet appeared and I was ignorant of Heffter's publication. The above mentioned enquiries were nugatory but they led to the present one.

in proliferating plant tissues. Buffa then showed that the same colour reaction is given by certain animal tissues.

In 1908 Heffter [1908] applied the nitroprusside test to a great number of tissues and tissue extracts and obtained positive results in many cases. Later, it would seem independently, V. Arnold [1911] showed that the reaction under proper conditions is displayed by practically all organised animal tissues. He at first described it as a protein reaction, though recognising that the plasma proteins gave a negative result.

A little later Arnold found that a strong nitroprusside reaction may be given by protein-free extracts of tissues, and came to the conclusion that free cysteïn was the responsible substance. Although he did not isolate cysteïn he considered that the evidence proved it to be a primary cell constituent in Kossel's sense.

Heffter, however, first gave definite form to the view that the presence of the HS— group, whatever its associations, may, owing to its labile hydrogen, be, in part at least, responsible for the reducing properties of protoplasm, and also, perhaps, indirectly for oxidations in the cell. Basing it upon known analogies he made the suggestion that during the autoxidation of the sulphydryl group peroxide of hydrogen may be formed and the peroxide oxygen then transferred, with or without the influence of a peroxidase, to other substances in the cell. If, further, HS— groups can be supposed to act continuously as promoters of cell oxidations, their own spontaneous oxidation to —S—S— groups must be reversible. In this connexion Heffter called attention to the fact that cystine can be reduced to cysteïn by sodium sulphite, and suggested that in the cell some substance might, like the sulphite, act as an "acceptor" for the oxygen of the water molecule, leaving the hydrogen of this to reduce the disulphide group once more to sulphydryl groups. Heffter's views have become generally familiar owing to the publication of an admirable discussion of the possibilities which underlie them by Thunberg in 1911.

Though very suggestive, these views have remained wholly theoretical. No experimental proof has been given of the existence of free cysteïn in the cell in the sense pictured by Arnold, nor has an HS— group been located in any other substance. Not without an isolation of the cell constituent actually responsible for the nitroprusside reaction, and a study of its properties, could Heffter's theoretical views receive experimental support.

Such an isolation is now to be described, together with a preliminary study of the substance from the standpoint of its possible functions in the reductions and oxidations of tissues. It proves to be a dipeptide, containing glutamic acid and cysteïn. It possesses properties which suggest that it may well play an important part in chemical events within the living cell. The methods of extraction have been such that there could be no possibility whatever of splitting off the dipeptide from other polypeptide material by hydrolysis.

## Section II. The Nitroprusside Reaction.

The nitroprusside test as applied by Heffter and Arnold consists in adding to tissue extracts, or to the fluid in which a piece of tissue is suspended, first a few drops of a moderately strong (5 %) solution of potassium nitroprusside and then excess of ammonium hydroxide. There can be no doubt that the use of ammonia is preferable to that of caustic alkali. But I have found that the colour reaction, as given by the tissue constituent to be described, is very greatly intensified when developed in the presence of a high concentration of certain ammonium salts, just as Rothera found to be the case when acetone or aceto-acetic acid is concerned. Doubtless the mechanism of the reaction is closely the same in each case, involving a reduction of the nitroprusside molecule. The colour produced resembles that of permanganate, and is less purple than when, under similar circumstances, ordinary sulphides act upon nitroprusside. If a small piece of fresh tissue, such as liver or kidney, be placed in, say, 5 cc. of a saturated solution of ammonium sulphate, two or three drops of a 5 % solution of sodium nitroprusside being then added, and finally excess of ammonium hydroxide, the tissue takes on a deep magenta colour. The reagents do not easily penetrate intact muscle fibres which should be disintegrated before applying the test. If the tissue be first heated with a little very dilute acid, acetic or other, the solution cooled, saturated with ammonium sulphate, and the test then applied, the colour will be found intensified. If the organ be ground up with sand it becomes maximal. From practically all the tissues examined the substance responsible for the colour reaction can be extracted by hot water and from such extracts it has been isolated.

## Section III. Isolation of a Dipeptide.

Yeast has been chiefly used as a source of the substance to be described, but the same product has been obtained from mammalian muscle, and mammalian liver.

There was every reason in advance to expect that it would be found to have a very low concentration in the tissues. As already stated, the nitroprusside reaction is extremely delicate, and tissue extracts when completely freed from proteins contain only very small amounts of organic sulphur. The actual yield of $H_2S$ when yeast cells or animal tissues are treated with sulphur (supposing this to measure sulphydryl groups with any accuracy) is minute.

The discovery of a method of isolation cost much labour; but could the nature and properties of the substance have been known in advance, greater difficulties might well have been expected.

Unfortunately the methods available for the separation of most types of native cell constituents are very limited. Fractional precipitation by salts of the heavy metals is at best clumsy, and as such precipitations are usually only in a very limited sense selective, it is difficult to make this method quantitative. Nevertheless after many efforts to reject it in favour of others,

it was finally adopted in the present endeavour. Needless to say, the basis of procedure was throughout largely empirical. The substance had, so to speak, to be teased out from its complex associations in the tissue. Fortunately the procedure, though tedious to describe and occupying much time in practice, is easy to repeat, and, given attention to details, invariably successful even in little practised hands.

*Method of separation.*

The separation from yeast may be first described. Minor modifications were made in the case of animal tissues which will be mentioned later.

Moist baker's yeast (45 kilos.) is boiled up with three successive quantities of tap water, large enamelled iron basins serving well for the purpose. For the above mentioned quantity of yeast about 10 litres of water may be used for each extraction. The extracts are filtered hot through Buchner funnels and the residue of the yeast well washed with hot water. The combined clear extracts are partially neutralised with dilute sodium hydroxide but left definitely acid to litmus. Neutral acetate of lead is then added until it ceases to precipitate. The precipitate is allowed to settle, the supernatant fluid syphoned off, and the precipitate then filtered off by use of several large Buchner funnels. It is very thoroughly washed with cold water.

The lead precipitate is next ground up in mortars and extracted with cold half-normal sulphuric acid until fresh extracts cease to give the nitroprusside reaction. With care this operation can be so conducted as to leave the lead sulphate free from the reacting substance, while the extracts contain relatively little free sulphuric acid. To the sulphuric acid extract, amounting usually to about 12 litres, uranium acetate is added until a little of the filtered fluid gives a strong brown colour with potassium ferrocyanide. Hot saturated barium hydroxide solution is next added to alkalinity and then in further excess until it ceases to produce a precipitate. The heavy precipitate is then filtered off at the pump and washed with cold water. At this stage a large proportion of certain polypeptide material originally precipitated by the lead, and all the phosphoric acid in the yeast extracts, are removed.

From the filtrate, which will measure perhaps 20 litres, barium is removed as sulphate, a small excess of sulphuric acid being left in the solution. The filtrate from the barium sulphate is now precipitated with acid mercuric sulphate solution[1], any large excess being avoided. The bulky mercury precipitate is well washed, suspended in water, and decomposed with $H_2S$. The filtrate and washings from the mercuric sulphide, freed from $H_2S$ by a current of air, are measured (1000–1500 cc.) and enough sulphuric acid added to make the mixture roughly half-normal in acidity. Phosphotungstic acid dissolved in half-normal sulphuric acid is then added in rather greater excess than is necessary to produce the maximum precipitation. At this stage there is greater danger than at any other of removing part of the product sought. In

[1] Prepared as originally used by Hopkins and Cole [1901] for the isolation of tryptophan.

higher concentrations it is precipitated by phosphotungstic acid, but at the dilution indicated the loss is small. Its phosphotungstic compound is more soluble in excess of the reagent than those of the substances from which it has to be freed. If a little of the solution is tested by boiling with lead acetate and sodium hydroxide before and after the precipitation with phosphotungstic acid the reduction in the blackening given by the loosely combined sulphur should be slight. I have so far found it impossible to dispense with the use of phosphotungstic acid at this stage. The precipitate is filtered off, phosphotungstic acid removed from the filtrate by means of barium hydroxide, and the latter afterwards removed as sulphate. At this stage the solution, which need not measure more than about 4 litres, is again precipitated with the mercuric sulphate reagent, a perfectly white precipitate being obtained. This should be decomposed, and the mercury sulphide washed at the pump, with the use of as little water as safety allows, in order to keep the concentration of the product as high as possible. If a little of the solution be thoroughly boiled to remove all traces of $H_2S$ it will be found to give an intense nitroprusside reaction, and a rich yield of sulphide when boiled with lead acetate and caustic alkali. The final treatment may be varied.

(*a*) In one method the copper compound may be used. The reduced dipeptide gives, like free cysteïn, a highly characteristic blue-grey precipitate with copper sulphate; but a more convenient method of separating it as a copper compound is to add to the solution from the above described second mercury separation, which contains a little free sulphuric acid, moist, freshly precipitated, copper hydroxide. When the vessel containing the solution is shaken the change from the appearance of the hydroxide added to that of the copper compound of the dipeptide is exceedingly characteristic. After the hydroxide has been added in excess, complete precipitation is secured by adding sodium hydroxide to the supernatant blue liquid until it is nearly but not quite neutral to litmus. The copper compound is decomposed with $H_2S$. The filtrate from the sulphide after removal of $H_2S$ is made just alkaline with baryta and a stream of air is passed through it until complete oxidation of the dipeptide has occurred as shown by the disappearance of the nitroprusside colour reaction. The solution is then carefully adjusted so as to be entirely free from both barium and sulphuric acid, concentrated under reduced pressure to a bulk of about 10 cc. and then poured into 100 cc. of absolute alcohol. It should stand under the alcohol until it becomes wholly anhydrous and friable, and is then filtered off at the pump.

(*b*) Otherwise the dipeptide may be separated at the final stage as the lead compound. Neutral lead acetate precipitates the substance from neutral solution only when the latter is in the reduced form, and then, in the case of the pure substance, not completely. If, however, lead acetate be added to the solution obtained on decomposing the above described second mercury precipitate, the greater part of the product is thrown out. The lead salt is decomposed with $H_2S$ and the solution treated exactly in the manner described

in the case of the copper compound. I have more than once thrown out in this way a first fraction with lead, and after removing lead from the filtrate have precipitated the remainder with copper. In such cases analyses of the two final products have closely agreed.

The substance is freely precipitated by silver salts, and completely by means of silver sulphate and baryta. I have not, however, found precipitation by silver of any advantage in purification. On a small scale one can dissolve the silver compound in hot water from which it separates on cooling. This seemed a characteristic property of which advantage might be taken; but on the large scale it is difficult to make use of it without danger of decomposition.

In making a preparation from animal organs the finely minced tissue should be allowed to extract for two or three hours with its own weight of water at 40°. The first extract is squeezed out through linen and the residue re-extracted. The extracts are finally mixed, filtered at the pump, the filtrates precipitated with neutral lead acetate and the procedure followed as with yeast. An alternative is to allow the tissue to stand with $N/10$ sulphuric acid (1 litre per kilo.) over night. The acid is nearly neutralised by the addition of 20 % caustic soda solution in small quantities at a time. The mixture is then heated and carefully neutralised as it reaches the boiling point. It is filtered at the pump and the residue extracted twice again by boiling with water. This latter method has generally been used for muscle. When it is employed it is, for some reason, advantageous to precipitate with uranium acetate at the first stage of treatment, instead of later. The reagent is added in saturated solution to the mixed extracts until it just ceases to produce a precipitate. The precipitate is filtered off and lead acetate applied to the filtrate. The sulphuric acid extract from the lead precipitate is subsequently precipitated with barium hydroxide alone.

The yield of the product from yeast has varied from 0·1 to 0·15 gram per kilo. From muscle it was about the same. The liver is, I think, undoubtedly richer in the substance, but in the one preparation made from it the weight of material taken was not recorded. Although the method of separation described is of course not quantitative, the figures given certainly represent the order of the amount actually present.

## Section IV. Properties of the Substance.

Evidence is given in later sections to show that the substance is present in the tissues in its reduced form, that is, as a dipeptide containing glutamic acid and cysteïn. When dissolved in this reduced form it is readily oxidised by atmospheric oxygen; most readily, as in the case of free cysteïn [Matthews and Walker, 1909], when its solutions are neutral or faintly alkaline. In the acid condition the HS— group of its cysteïn moiety is much more stable toward molecular oxygen. When in the oxidised form its solution can be easily reduced by zinc and sulphuric acid or by sodium sulphide. It will be

shown later that it is specifically reduced by tissue agencies. Needless to say the processes of reduction and oxidation can be easily followed by the use of the nitroprusside reaction, of which the oxidised form gives no trace.

As each molecule of the dipeptide contains only one HS— group oxidation, in producing the disulphide (—S—S—) grouping, must involve a linkage of two molecules. Preliminary molecular weight determinations have borne out this assumption.

Nearly all the final preparations of the substance have been made as described in the last section in the oxidised form, and this because in the final manipulation it is difficult to avoid partial oxidation of the reduced substance. It has proved impossible in the case of the oxidised dipeptide, which is a substance of high molecular weight (498), to obtain it crystalline, or to prepare a convenient crystalline derivative. I have recently found however that if all precautions are taken to avoid oxidation and the substance is separated while reduced, a crystalline preparation can be obtained. The dipeptide in this form is somewhat freely soluble in alcohol so that a mixture of alcohol and ether was used for its final precipitation (cf. last section). After it had stood under the mixture for three days its solubility in water was considerably reduced. When dissolved in hot water it separated on cooling as thorn-apple crystals which upon recrystallisation were replaced by acicular aggregates. The facts suggest that the dipeptide while standing under alcohol-ether is probably converted into the diketopiperazine anhydride. Advantage will be taken of this observation in future work.

The oxidised substance as employed for the analyses given in the next section is a snow-white non-hygroscopic powder. If heated quickly it softens at 165°–167°. At 182°–185° it melts with evolution of $CO_2$ and the melt runs up the tube. All its preparations have behaved in the same way either when separate or mixed.

It is exceedingly soluble in water, but apparently in no organic solvent. Its behaviour towards precipitants will have been sufficiently indicated in the previous section.

It is easily hydrolysed by mineral acids yielding glutamic acid and cysteïn, but it appears to be wholly resistant to the proteolytic ferments of the tissues. The effect of trypsin has not been studied.

Solutions of the reduced dipeptide when shaken with sulphur give off $H_2S$.

## Section V. Evidence of Constitution.

Before it was actually obtained in substance, I was led to expect that the compound to be isolated might have the constitution of a dipeptide. By the use of nitrogen determinations made in solutions obtained at various stages of fractionation it was observed that constancy was arrived at when the amino-nitrogen estimated by van Slyke's method was about half the total nitrogen, and when the former was doubled after boiling the solution with mineral acids.

At such a stage the ratio of sulphur to nitrogen also proved constant. It should be noted that for a reason already mentioned most of the analytical studies have been made upon the oxidised product. As was pointed out in the last section, oxidation (as in a current of air) should involve the production of a disulphide grouping which must link two dipeptide molecules together and so nearly double the molecular weight of the product. This, of course, will not affect the results of hydrolysis, except that cystine should be obtained instead of cysteïn.

*Hydrolysis.*

(*a*) *Separation of glutamic acid.* Two grams of the oxidised product from yeast were boiled for 8 hours with 100 cc. of 25 % aqueous hydrochloric acid. The solution when concentrated under reduced pressure to about 10 cc. and cooled, deposited crystals which resembled those of the hydrochloride of glutamic acid. They were filtered off through a small linen filter, and washed at the pump with strong HCl. The filtrate was saturated ice-cold with HCl gas, and stood in ice over night. Another crop of crystals was filtered off, and the filtrate again concentrated and again saturated with HCl. A third crop was similarly obtained, all three crops being then united, dissolved in a minimal quantity of water, and again saturated with HCl at 0°. The crystals were again filtered off and the filtrate mixed with the original filtrates. The mixed filtrates on standing for a week deposited a further fraction of a hydrochloride. By careful treatment a final combined product was obtained which had all the characters of pure glutamic acid hydrochloride. It weighed 1·06 g. or 53 % of the product hydrolysed. A dipeptide of cysteïn and glutamic acid would have a molecular weight of 250, and the corresponding disulphide compound one of 498. The latter would yield 59·03 % of glutamic hydrochloride. The above yield from 2 g. of product is therefore equal to 90 % of theory.

The melting point of glutamic hydrochloride is often given as 198°. I have on previous occasions found it to be higher than this. A very pure carefully analysed product made from wheat proteins by Dr H. Raistrick showed softening at 200° and melted at 206° (uncorr.) with decomposition. Heated side by side with this, the product prepared as above behaved in an identical manner, and a mixture of the two gave precisely the same melting point.

Two other preparations, from different supplies of yeast, similarly treated, have given similar results. I have found that glutamic hydrochloride, being so highly dissociated in aqueous solution, will, when dissolved in a minimum of water and its solution mixed with six to eight times its bulk of alcohol, slowly yield crystals of free glutamic acid. This is a convenient method of preparation when one is working on a small scale. Small quantities of the hydrochloride prepared from the hydrolysis of yeast products were treated in this way side by side with a pure preparation from wheat gliadin. The crystals

in each case were identical, having the characteristic rhombic octahedral form. When filtered off, washed and dried, they melted in every case at 192° (uncorr.).

The nitrogen of two preparations of the hydrochlorides was determined by Kjeldahl.

Found (*a*) 9·47 %, (*b*) 9·48 %. Calculated 9·52 %.

(*b*) *Separation of cystine.* Filtrates from the glutamic acid preparations after evaporation on the water bath to remove HCl (the residues being subsequently dissolved in dilute sulphuric acid and precipitated with mercuric sulphate) have in all cases yielded cystine. For quantitative work, however, the original products were hydrolysed with sulphuric instead of hydrochloric acid.

Two grams of a yeast product were boiled for eight hours with 25 % sulphuric acid. The solution was then diluted with four times its volume of distilled water and precipitated with the same mercuric sulphate solution as that employed for the separation of the original product. A heavy white precipitate was produced which was filtered off and washed at the pump. The filtrate gave no sulphide when boiled with lead acetate and caustic soda. The precipitate was decomposed with $H_2S$, the filtrate from the mercury sulphide was freed from $H_2S$ and sulphuric acid then removed by a slight excess of barium hydroxide. While still just alkaline it was aerated for some hours until the nitroprusside reaction had disappeared. Excess of baryta was then exactly removed. On standing without evaporation the solution (*circa* 250 cc.) deposited a small amount of cystine in typical hexagonal plates. On concentration to small bulk a white product separated which under the microscope appeared to consist of acicular aggregates. From the mother liquors of this fraction a second yield of colourless product was obtained on further evaporation. The two together weighed 0·59 g. or 60 % of theory. Although mercuric sulphate precipitates cystine from acid solutions with great completeness, it is, for some reason, difficult to recover the product quantitatively. My colleague Dr T. S. Hele, who, some years ago, studied the methods for separating this amino-acid very carefully, tells me that when he precipitated pure cystine solutions by mercuric sulphate and the cysteïn which resulted on decomposing the precipitate with $H_2S$ was allowed to oxidise in the air, the amount of cystine recovered by him was of the order of 60 % only. As all who have experience of the matter are aware, the quantitative separation of cystine from other amino-bodies is always difficult[1].

Cystine was obtained in a similar manner after hydrolysing other preparations from yeast. Three such products were combined, dissolved in hydrochloric acid, and the perfectly clear solution neutralised with ammonia. The cystine separated in uniform typical hexagons.

0·3596 g. was dissolved in 25 cc. of 10 % HCl.

[1] For an experience with regard to the destruction of cystine during acid hydrolysis see van Slyke [1911].

In an 18·9 cm. tube it gave with the sodium flame a laevo-rotation of 5·45°.

$$[a]_D = -200°.$$

The specific rotation of *l*-cystine from proteins and keratin is usually given as − 205°. The high rotation distinguishes cystine from other amino-acids, and the above figure indicates that the substance dealt with is normal *l*-cystine.

The nitrogen was determined by a micro-Kjeldahl method, the apparatus and reagents being controlled by blanks and by estimations made upon a specimen of pure cystine from hair.

(*a*) 11·39 %, (*b*) 11·56 %. Calculated 11·66 %.

Sulphur was estimated by means of a micro-Carius method which had previously given good results with similar quantities of pure cystine from hair.

0·067 g. gave 0·1169 g. $BaSO_4$ = 26·45 % S (calculated 26·45 %).

After the separation of cystine from the products of hydrolysis and removal of the sulphuric acid the glutamic acid was either separated as the hydrochloride or precipitated by mercuric acetate and sodium carbonate and then liberated as the free acid. In all cases the properties of the product were those of normal *d*-glutamic acid. The final residues from several preparations of hydrolysed material (the glutamic acid and cystine having been in each case removed on the lines described) were combined, after removal of HCl when necessary. No trace of any other amino-acid could be obtained from these residues by crystallisation or otherwise. A minute crystalline fraction proved to be glutamic acid, and the mother liquor from this gave no more than a very small gummy residue with a slight mercaptan-like odour.

*Distribution of nitrogen.*

In the case of a compound suspected of being a simple peptide good evidence of constitution should be obtained by comparing with the total nitrogen the amino-N before and after hydrolysis. When, however, a substance contains cystine it must be remembered that this amino-acid gives abnormally high values in determination by van Slyke's method. Allowing for this fact the substance under description in this paper gives on these lines satisfactory evidence of being a dipeptide.

In the case of cystine van Slyke obtained the values 12·52 and 12·68 for the percentage of amino-nitrogen; theory requiring 11·67. With the reagents employed in my laboratory, however, which gave normal values for other amino-acids, considerably higher values than these were got for pure cystine and also for cysteïn. In a consistent series of determinations the value 14·5 was obtained for both substances. As the precise reason for the abnormal behaviour of these thio-acids in van Slyke's process is unknown it is not quite clear how a simple peptide containing one of them would behave, and a correction based on the above values would not necessarily be satisfactory.

Somewhat high values for the amino-N should at any rate be expected. A product from yeast gave the following figures:

Total N 11·9 %; amino-N 6·8 %; amino-N after hydrolysis 13·3 %.

If a calculated addition to the theoretical figures can be based on the data just given for cystine, and if it be applied to the cystine half of the molecule only, the amino-N for the oxidised product would, on the assumption that it is derived from a dipeptide, be 6·3 %, and, after hydrolysis, 12·6 %.

It is unfortunate that the properties of cystine intrude in the estimations, but in the case of a substance which on hydrolysis yields two amino-acids, and two only, and yields an amount of amino-nitrogen agreeing so closely with what special considerations would lead one to expect—this amino-nitrogen being doubled on hydrolysis—there can be no doubt that a dipeptide is in question. In the case of even a tripeptide, if it contain only monamino-acids, the amino-nitrogen must be trebled after hydrolysis and the increase grows, of course, proportionally greater with increase in the number of constituent amino-acids. Other products from yeast besides the above have given quite similar figures for amino-nitrogen and the doubling on hydrolysis has proved to be a constant happening.

*Elementary analyses.*

The analyses have, for a reason already discussed, been made nearly always on the oxidised product, but except in the case of hydrogen, the effect of the oxidation upon percentage composition is of course extremely small. The dipeptide $C_8H_{14}O_5N_2S$ with a molecular weight of 250 would yield with loss of two H atoms a disulphide derivative of molecular weight 498. In the case of such a substance the evidence for constitution to be got from elementary analyses is of less importance than that based upon facts such as have been already discussed.

As therefore the material costs so much labour to prepare there was a temptation to make the analyses on quantities as small as possible, though the presence of sulphur and the consequent necessity of using lead chromate made it undesirable to rely upon micro-combustions.

The results agree satisfactorily with the composition of a dipeptide having the constitution suggested by the other evidence. The nitrogen figures have, it is true, been persistently somewhat high. This is probably due, in part at least, to the presence of small amounts of ammonia in the final product. It will be remembered from the description of the preparations that immediately before evaporating the final solutions they were oxidised by aeration while only barely alkaline from barium hydrate. The process involves the passage of many litres of laboratory air, and from lack of realisation of the danger no attempt was at first made to trap off ammonia. Once the slight excess of baryta had been removed the acid nature of the free substance would have prevented removal of ammonia during evaporation. On an occasion when the above process was omitted and the product prepared for analysis in a reduced

form the theoretical figure was obtained (11·25 %) for nitrogen. The following complete analysis was made with the use of normal quantities of material. To other preparations micro-methods have been applied.

The sulphur was estimated by fusion with sodium peroxide and the nitrogen by Kjeldahl.

Product from yeast finally purified through the lead compound:

Found C, 38·21; H, 5·12; S, 12·31; N, 11·70.

Calculated C, 38·55; H, 5·22; S, 12·85; N, 11·24.

Five other determinations of nitrogen were made upon different products, all by a micro-Kjeldahl method. Of these the first two high results were obtained early in the research; the others after more experience had been gained.

Nitrogen % (12·37, 12·10); 11·68, 11·55, 11·37 (calculated 11·24).

The following results for sulphur were obtained from diverse products. All determinations had to be made upon small quantities of material—from 0·060 g. to 0·100 g.

S = 12·30, 13·22, 11·68, 12·70 %.

Mean = 12·40 %; calculated 12·80 %.

All the data so far given apply to products from yeast. A preparation from ox-muscle had exactly similar properties and gave 11·75 % N by Kjeldahl. After hydrolysis the glutamic acid, as obtained pure, amounted to 88 % of the theoretical yield. Cystine was identified but not estimated. A small preparation from liver (0·4 g.) had again identical properties. When it was mixed with a preparation from yeast the behaviour on heating was unaffected. Glutamic acid and cystine were fully identified in the products of hydrolysis but they could not be estimated.

There can be no doubt from the evidence that the compound isolated is, in its reduced form, a dipeptide of which the constituents are glutamic acid and cysteïn. I have not yet determined the relation of one amino-acid to the other. An endeavour to do this is now in progress.

Until the constitution is finally established it may be premature to suggest a name for the substance. But, provisionally, for easy reference, the name *Glutathione* will perhaps be admissible. It leaves a link with the historic *Philothion*, has the same termination as *Peptone*, which has long served as a name for the simpler peptides, and is a sufficient reminder that the dipeptide contains glutamic acid linked to a sulphur compound.

## Section VI. Distribution.

The statement of Gola that the nitroprusside reaction is given by proliferating plant tissues I have confirmed in many instances, but its intensity is usually far less than in animal tissues. The few species of bacteria examined have all displayed it. In the animal it is given by all the tissues tested except in the case of connective tissues. Blood plasma and serum give, as Arnold found, no trace of the reaction. It is characteristic indeed of cellular tissues

with active metabolism. The constituents of the fowl's egg in native condition give no trace, but a thirty hours' embryo if observed under a dissecting microscope will, especially if first flooded with a little weak acetic acid, be seen to give a vivid reaction throughout its mass.

I cannot pretend to have explored the zoological field at all thoroughly but have obtained the reaction from tissues and tissue extracts of the following as types: muscle and other tissues of the earthworm; adductor muscle of the oyster (in most tissues of this mollusc the reaction is very slight); muscles of the blow-fly; muscles and other organs of the lobster; muscles of the skate and of the cod; other organs of fish not examined; all organs of the frog; all organs of every mammal examined. Except in the case of the muscles the reaction is much less intense in cold blooded animals than in warm. It is always possible, however, that this is a matter of tissue equilibrium; more of the substance may be in the oxidised form.

It would of course be going much too far at present to claim that the display of the nitroprusside reaction proves the presence in all these localities of the substance described in this paper. It may in different groups of animals be represented by different if analogous substances; but it is noteworthy that the cause of the reaction is the same in structures so diverse as the yeast cell and mammalian organs such as muscle and liver.

With regard to pathological relations it may be stated that actively growing cancer cells seem to show a nitroprusside reaction of surprisingly low intensity; but I have seldom had the opportunity of examining growths soon after their removal from the body.

## Section VII. Physiological Relations.

Observations will now be briefly described which seem to show that the dipeptide exercises real functions in the chemical dynamics of the cell. An attempt to relate its properties more completely with other known facts and to current theories concerning tissue respiration will be made in a later communication.

As already stated the dipeptide, when in neutral or slightly alkaline solution, spontaneously oxidises in air at ordinary temperatures though more slowly than does pure cysteïn. As Bach [1912] has pointed out the uptake of molecular oxygen by a substance undergoing autoxidation must by Ostwald's law of successive reactions necessarily involve preliminary peroxide formation. The less stable state, say $R\begin{matrix}O\\|\\O\end{matrix}$ + R, however brief its duration, must precede the more stable state R : O + R : O. It is therefore not without significance in any case to know that a definite cell constituent can be isolated which belongs to the class of autoxidisable substances. The existence of such, in some form or other, is assumed in accepted theories concerning oxidase systems.

But such oxidase systems, able to induce limited oxidations in constituents

which do not themselves react with molecular oxygen, are not the only type of oxidative mechanisms represented in the cell. Indirect oxidations induced by the action of catalysts in the presence of suitable hydrogen acceptors have recently received much attention, and would seem to play a very real part in biological phenomena. Bach has very fully studied what he calls "hydrolytic oxidation-reduction" reactions in which, under the influence of such catalysts as metals of the platinum group, the reduction of one substance by the hydrogen ion of water is associated with the simultaneous oxidation of another substance by the hydroxyl ion. Such a case, with an organic catalyst taking the place of the metal, is the well-known Schardinger reaction of fresh milk. Milk neither reduces methylene blue nor oxidises an aldehyde, but if both are present the former substance acts as a hydrogen acceptor and the latter as an oxygen acceptor and simultaneous reduction and oxidation are the result. Bach has shown that the same or a similar catalyst is present in the tissues.

A catalytic mechanism different from, though closely related to that just mentioned, may also exist in the cell, unless indeed as in the view of Wieland [1914] it is a more general mechanism covering the supposed activities of those already mentioned.

Wieland sees the essential cause of certain typical biological oxidations not in the activation of oxygen but in the activation of hydrogen. The proof that this activation occurs renders unnecessary he thinks any attempt to give a general application to the Engler-Bach peroxide theory. On the other hand Wieland's contention somewhat modifies Bach's conception of hydrolytic oxidation-reduction.

Starting with the conception of a catalyst, which acts as a "dehydrase" and confers lability upon hydrogen atoms, the assumption that oxydases, reductases, and "mutases" separately exist becomes from Wieland's standpoint superfluous.

His conceptions are mainly supported by a careful quantitative study of the action of Schardinger's ferment on salicylaldehyde. The aldehyde is assumed to act in the form of its hydrate (an assumption which would seem after all to negative any wide divergence between the views of Wieland and Bach). The enzyme activates two of the hydrogen atoms which are then made available for an acceptor. This acceptor may be (1) molecular oxygen, (2) such reducible substances as are represented by methylene blue, or (3) in a particular case the acceptor may be a molecule of the aldehyde itself in the unhydrated form. As an end result we have, in case (1) a frank oxidation of the aldehyde; in case (2) an observed reduction associated however with indirect oxidation of the aldehyde; in case (3) on the other hand the Cannizzaro reaction—the so-called "mutase" effect:

$$R.CH(OH)_2 + R.CHO = R.CO_2H + R.CH_2OH.$$

Whether any one of these end results is attained, or a mixture of all three, depends (as Wieland's quantitative results seem to show) upon the concentra-

tion and speed of reaction of the various acceptors. The most important hydrogen acceptor for energy production in the cell is clearly oxygen, but the reduction of other acceptors may be equally important for other aspects of cell activity.

Thunberg [1920] has accepted Wieland's conception of the events and in a remarkable paper recently published he has used the methylene blue technique to supply evidence as to the probable nature of intermediary metabolites. Tissue (muscle) deprived of its power of reducing methylene blue by thorough washing with water but still containing the catalyst is suspended in a weak solution of the pigment. Small quantities of the substances under investigation are then added. The methylene blue acts as hydrogen acceptor. If the second substance can act as a hydrogen "donator" then the dye is reduced. The ferment acts as a hydrogen "transportase." Only a limited selection out of a large number of substances tried could induce by their presence the reduction in question, and that they should do so is taken by Thunberg for presumptive evidence that they are—or are closely related to—products which appear in normal intermediary metabolism.

I have said this much to indicate the importance which is now attached to the presence of acceptors, and especially of hydrogen acceptors, co-existing with substances to be oxidised. The same importance is attached to hydrogen acceptors in fermentations.

The dipeptide when in the disulphide (oxidised) form can act of course under conditions as a hydrogen acceptor, and the labile hydrogen of the resulting sulphydryl group, under other conditions, as an oxygen acceptor.

As already remarked, however, dynamic functions in the cell cannot be claimed for the substance unless it can be shown that its oxidation and reduction are brought about in a reversible manner by factors present in the cell itself.

That fresh tissues possess a reduction potential such as to induce rapid reduction of the substance can be at once shown by the simplest technique. In one test tube are placed a few cc. of distilled water and in another an equal amount of a weak solution of the dipeptide in its oxidised form which gives of course no nitroprusside reaction. A small weighed piece of fresh tissue (liver, kidney, muscle, etc.) is then dropped into each. If the tubes be then placed in a bath at 35° it will be found that the fluid of that containing the added dipeptide will, after an hour or two, give the nitroprusside reaction with an intensity out of all proportion to the fluid of the control tube. The latter may give a slight reaction due to diffusion from the tissue of preformed reduced dipeptide. Under anaerobic conditions the change goes faster. Antiseptics may be used. The reduction may be followed in its stages by placing crystals of ammonium sulphate mixed with nitroprusside on a tile. A couple of drops of the fluid followed by one of ammonia will show the increasing intensity of the colour reaction. It is difficult to obtain the reducing system in solution; but the dry powder obtained by grinding a tissue with sand under alcohol,

washing the residue with fresh alcohol on a filter, and allowing it to dry in the air, reduces efficiently. Such a powdered preparation from liver, deprived so far as possible of connective tissue, has served well for quantitative observations.

It is next easy to show that conditions exist on the other hand in which factors present in tissues can promote oxidation of the reduced dipeptide. It is a circumstance familiar to all who have studied the reducing power of excised tissues, by the use for instance of methylene blue as an indicator, that the reduction potential rapidly falls off as survival processes progress. It must of course be remembered that conditions for reduction and oxidation vary with the substance concerned. In the case of the dipeptide survival changes involve a complete reversal of the existing relations. A tissue which has stood under aseptic conditions for a sufficient time fails to exhibit the nitroprusside reaction and it can be shown without difficulty that conditions then develop under which the reduced dipeptide is oxidised. As this oxidation goes on anaerobically, some other substance must now act as a hydrogen acceptor. Especially upon actual autolysis does this change in the equilibrium of reducing and oxidising agencies occur. If disintegrated tissues be suspended in four to five times their weight of chloroform water and allowed to autolyse in corked flasks at 37° the fluid during the first few hours will show a strong nitroprusside reaction. This gradually fails in the fluid, and, ultimately, after a period varying with the particular tissue, also in the incompletely autolysed tissue fragments. The end-point occurs soonest with the kidney; more slowly with liver, because of its original high content of the reduced dipeptide; and still more slowly in the case of muscle. If, when the colour reaction has ceased, a further quantity of reduced dipeptide be introduced so as to restore a strong reaction to the fluid, this will be found also to disappear on standing. The process goes on with undiminished velocity, and indeed seemingly faster, under anaerobic conditions. That oxidation has occurred is shown by the fact that the process is easily reversible by reduction. If some of the fluid which has ceased to give the colour reaction, and especially if its content of dipeptide had been increased on the lines mentioned, be boiled, filtered, and placed when cool over a piece of perfectly fresh tissue an intense nitroprusside reaction once more develops.

The significance of this change of oxidation potential during survival and post-mortem changes need not be here discussed. For the moment I am concerned only to show that there exist in tissues mechanisms for both reduction and oxidation of the dipeptide. If *in vitro* the one mechanism has to disappear before the other can be displayed, it is yet perfectly probable that in the organisation of the cell both mechanisms are employed.

Considerations may now be put forward which indicate that the dipeptide does as a matter of fact play a real part in cell dynamics.

Fresh tissues of course reduce methylene blue, and so, when itself in the reduced condition, does the dipeptide. But as has just been shown the former

also reduce the latter. It would seem then that a mechanism in the tissues has a greater reduction (or lower oxidation) potential than the HS— group of the dipeptide. As a matter of fact, however, the exact relations depend upon the hydrogen ion concentration of the medium. It should be remarked that the reduced form of the dipeptide is more stable on the acid side of neutrality. The following facts are of interest.

If the substance in the oxidised (disulphide) form be added to a solution of methylene blue in contact with fresh tissue, then, if the reaction of the system is even slightly on the acid side of neutrality, say at $P_H = 6{\cdot}8$, the reduction of the dye is greatly slowed, as comparison with a control preparation will at once show. The dipeptide acts here simply as a hydrogen acceptor and competes with the methylene blue in this respect, delaying or (according to its concentration) even preventing the reduction of the latter. If, however, the reaction of the medium be adjusted to $P_H = 7{\cdot}4$, or made very slightly more alkaline than this, the relations change. The normal rate of reduction of methylene blue by the tissue used is then markedly *accelerated* by the addition of the *oxidised* dipeptide. The phenomena are best observed under anaerobic conditions and the observations should be made in test tubes which can be evacuated, or which are corked and so fitted with glass tubing that the air can be replaced by pure nitrogen. As the tissues contain their own supply of the substance the contrast between the behaviour of a preparation to which dipeptide has been added and a control preparation is greater if the tissue used is first washed several times with sterilised distilled water. This removes a considerable part of the pre-existing dipeptide especially from the surface of the tissue. The tissue powder from alcohol, as described above, acts well and may also be washed before use[1]. Although I think the fact is not commented upon by authors who have used washed tissues in connexion with methylene blue reductions the concentration of the enzyme is reduced by extraction with water. In the experiments under discussion washing increases the contrast, but may increase the actual time required for reduction. The following data will illustrate what has been said. In the experiments given the tissue preparation was in each case the liver of the rat, ground under alcohol, air dried, and afterwards washed with distilled water. Similar results have been obtained, however, with other tissues, washed and unwashed. The first comparison shows the reversal in the effect of added dipeptide when the reaction of the system changes. The tubes were filled with nitrogen.

| Tissue preparation | Oxidised dipeptide added | Methylene blue 1 : 5000 | Water | $P_H$ at beginning | Reduction time |
|---|---|---|---|---|---|
| 0·5 g. | 0 | 0·5 cc. | 5 cc. | 7·5 | 3 hrs. 35 mins. |
| ,, | 4 mg. | ,, | ,, | ,, | 1 hr. 30 mins. |
| ,, | 4 ,, | ,, | ,, | 6·2 | 5 hrs. 20 mins. |
| ,, | 8 ,, | ,, | ,, | ,, | 15 hrs. + |

[1] It is noteworthy that the tissues of small animals such as the rat show under all circumstances a greater reducing power than those of larger animals.

The following shows the acceleration of reduction due to the addition of oxidised dipeptide to the system in neutral or slightly alkaline conditions. The solution in each case was a phosphate buffer solution, the $P_H$ being adjusted after the addition of the dipeptide. Chloroform was added to all the tubes and there was a layer of toluene on the surface. The tubes were filled with nitrogen.

| Tissue preparation | Buffer solution | Methylene blue | Oxidised dipeptide added | $P_H$ | Reduction time |
|---|---|---|---|---|---|
| 0·2 g. | 5 cc. | 0·5 cc. | 0 mg. | 7·4 | 5 hrs. 45 mins. |
| ,, | ,, | ,, | 4 | ,, | 2 ,, 40 ,, |
| ,, | ,, | ,, | 0 | 7·8 | 3 ,, 45 ,, |
| ,, | ,, | ,, | 4 | ,, | 1 hr. 40 ,, |
| 0·5 | ,, | ,, | 0 | ,, | 2 hrs. 30 ,, |
| ,, | ,, | ,, | 4 | ,, | 0 hr. 55 ,, |

The conditions can be adjusted to give more rapid reduction and sharper contrasts than the above.

| Tissue | Buffer solution | Methylene blue | Oxidised dipeptide | $P_H$ | Reduction time |
|---|---|---|---|---|---|
| Liver ·15 g. | 5 cc. | 0·3 cc. | 4 mg. | 7·4 | 0 hr. 25 mins. |
| ,, | ,, | ,, | 0 | ,, | 6 hrs. + |
| Muscle (washed) 0·2 g. | ,, | ,, | 8 | ,, | 0 hr. 10 ,, |
| ,, ,, | ,, | ,, | 0 | ,, | 3 hrs. + |

It seems clear that in the reactions described the —S—S— group of the oxidised dipeptide acts first as a hydrogen acceptor and under conditions of even slight acidity the resulting HS— groups are too stable to transfer the hydrogen to another acceptor. In neutral or slightly alkaline solution on the other hand the hydrogen is transferred to the methylene blue. If this view be the right one we have to recognise the important fact that the two reactions involved in the transference of hydrogen to the disulphide group under the influence of a tissue enzyme, and its subsequent transference from sulphydryl groups to the methylene blue acceptor, together run faster than the single reaction in which the dye is directly reduced by the tissue enzyme. The dipeptide then possesses what are essentially catalytic properties and would be fairly spoken of as a co-enzyme. Indeed, if tissues are very thoroughly washed, as in the observations of Thunberg referred to earlier, so that their power to reduce methylene blue is practically removed, the restoration of this power when the oxidised dipeptide is added at once gives the impression that it has the function of a co-ferment exerted on the lines suggested.

Thunberg's work, however, suggests of course another possibility. He found as already stated that a number of substances such as succinic acid, malic acid, and the like, including, as is noteworthy, glutamic acid, can, under defined conditions, restore reducing power to tissues deprived of it by washing. It is possible therefore that the dipeptide in the experiments just described was when in neutral or alkaline solution merely acting as a substance of this class. I am convinced however that this is not the case. The

compounds which act as hydrogen "donators" in Thunberg's experiments undergo an irreversible oxidation due to the removal of hydrogen atoms attached to carbon. I have evidence to show that this does not occur in the case of the dipeptide. Oxidation and reduction of this substance by tissue systems involve the thio-group alone and the strictly reversible change

$$—SH \quad HS— \rightleftharpoons —S—S— + H_2.$$

It is for this reason that the substance may be justifiably supposed to have definite and permanent functions in the cell.

The question arises whether the dipeptide has relations with the respiratory substance of Meyerhof [1918]. Preliminary observations have shown that it definitely increases the uptake of oxygen by washed yeast and washed muscle, but quantitatively the effect is small compared with the results described by Meyerhof himself. I have not yet tested its effect upon the reducing power of washed yeast on the lines of Harden and Norris's [1914] experiments.

A more complete study of its behaviour in such connexions is in progress.

There can be little doubt that the chief significance of the occurrence of cysteïn in the dipeptide, rather than free, lies in the fact that it is thereby protected from metabolic breakdown. Why, since both the glutamic acid and the cysteïn contained in it are the physiological forms of these amino-acids, the dipeptide should be resistant is not fully clear; but there is no doubt of the fact.

## Summary.

A substance responsible for the nitroprusside reaction which is given by nearly all animal tissues, and was applied by Heffter and by Arnold in proof of the presence of sulphydryl groups in the cell, has been isolated from yeast, from muscle, and from mammalian liver. It has the properties of the philothion of de Rey Pailhade.

Evidence is given to show that the substance is a dipeptide containing glutamic acid and cysteïn. The relation of the two amino-acids in the molecule has not yet been determined. Though present in low concentration (0·01 to 0·02 % of the fresh tissue) the dipeptide contains practically the whole of the non-protein organically bound sulphur of the cell.

The substance is autoxidisable, and, owing to the changes in the sulphur group of its cysteïn moiety from the sulphydryl to the disulphide condition and *vice versa*, it acts readily under varying conditions either as a hydrogen acceptor or an oxygen acceptor (hydrogen "donator"). It can be both reduced and oxidised under the influence of factors shown to be present in the tissues themselves.

Evidence is discussed which suggests that the substance has actual functions in the chemical dynamics of the cell.

The work described was begun with the assistance of Mrs E. C. Bulley. Later I had help from Miss Dorothy Foster and more recently from Mr H. F. Holden. I am indebted to my assistant, E. J. Morgan, for laborious manipulations carefully carried out.

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# XXXIII. THE INFLUENCE OF THE NERVOUS SYSTEM ON THE EXCRETION OF CREATININE.

## EXPERIMENTS ON NERVOUS AND MENTAL PATIENTS.

By ABRAHAM ALBERT WEINBERG.

*From the Physiological and Psychiatrical Laboratories, University of Groningen, Holland.*

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Since Folin's [1904, 1] method for estimating creatinine in urine became known numerous experiments have been conducted to determine the factors, which influence the excretion of this substance. Folin [1905, 1906] found that its amount in the urine stands in relation to the body-weight, and in such a manner that fat persons excrete a little less than others per kilogram of body-weight; he also found that creatinine is a product of endogenous metabolism. These results were verified by Van Hoogenhuyze and Verploegh [1905, 1908]; later Shaffer [1907, 1909] expressed the view that the degree of muscular development and more particularly muscular tonus has a bearing on the urinary creatinine. He found, however, that rapid muscular movements and fatigue have no influence on the excretion, and this has been confirmed by many observers. By a series of very interesting experiments Pekelharing and his colleagues [1910, 1911, 1913, 1916] observed an increased production of creatinine with increased muscular tonus and a decreased production with decreased tonus, and also a corresponding greater or smaller excretion of creatinine in the urine. Their view, namely, that muscular tonus is the most important factor in the excretion of creatinine, is shared by far the greatest number of observers.

The original object of my research was to determine the amount of creatinine in the urine of nervous patients. In connexion with this generally accepted tonus theory, it appeared to me to be of importance to make a systematic inquiry into the question, whether there is a smaller excretion of creatinine per kilo. of body-weight in the case of diseases with hypotonic muscles, than there is when the diseases are accompanied by an increased tonus of the muscles.

Earlier researches on the excretion of creatine and creatinine in nervous diseases have been performed by Spriggs [1908], Levene and Kristeller [1909], Skutetzky [1911] and others. These observers concluded that the excretion of creatinine in tabes dorsalis, spastic paralysis and paralysis agitans is more or less normal, whilst there is a decrease in the urinary creatinine in diseases

where much of the muscular tissue is destroyed, as for instance in the case of progressive muscular atrophy. Folin [1903, 1904, 2], Benedict and Myers [1907] and to a less degree Van Hoogenhuyze and Verploegh are responsible for a large number of experiments on psychical patients.

The first observers found normal or lowered values for the amount of excreted creatinine, but Van Hoogenhuyze and Verploegh found variations in this amount. They could not, however, say whether these variations went parallel with the frame of mind or not; but in general there was an increased excretion of creatinine with abnormal cheerfulness.

*Experimental Method.*

The patients were put on a creatine- and creatinine-free diet and this was continued for six days. In the cases where the patients were not necessarily confined to bed they were carefully guarded from tiring themselves. The amounts of urine of the last three days of the experiment were used to determine the average excretion of creatinine. The nurses took care that no urine was lost, and in all cases where the loss of urine was considered to be of such a nature as would influence the results, the experiment was looked upon as a failure. The amount of creatinine was usually ascertained as soon as possible; but when this could not be done, as happened on some days, small quantities of the experimental urine were preserved in the ice-chest until the next day.

The creatinine was determined according to Folin's method as applied by Autenrieth and Müller [1911] with the colorimeter of Autenrieth and Königsberger.

*Observations.*

As it would take me too long, I do not propose to give a detailed account of the 73 patients, who were employed in the experiments. I wish, however, to state that the cases, of which I do not treat, produced nothing that is contrary to the issues cited.

*Values with normal persons.*

It appeared to me to be undesirable to compare the values obtained with those that have up to now been considered normal.

Except in a few cases, the excretion of creatinine in pathological conditions has, so far, not been compared with that of normal persons under circumstances exactly similar to those of the patients experimented on. I have therefore to get a measure of comparison examined the urine of seven persons, who were in the university hospital at Groningen. The other patients were also in this hospital. The doctor in charge and myself carefully selected these people, and only those were taken, who had no nervous or mental disorder. In addition to this they were free from fever and the choice was limited to patients whose diseases were considered to be of no influence on the metabolism; and further to patients who willingly and without aversion took the creatine- and creatinine-free diets. This last point holds good for all the patients

examined. Of the seven, who were chosen, four were women and three men. The diagnoses were as follows:

A person cured from amentia (acute hallucinary perplexity).

Four persons with ulcus ventriculi.

A person almost cured from tuberculous peritonitis.

A person with a fractura colli femoris subcapitalis.

The daily quantity of excreted creatinine per kilo. of body-weight was respectively: 5·5, 5·8, 5·9, 6·2, 6·4, 6·6, 7·9 milligrams, and this gives a daily average of 6·3 mg. per kilo. of body-weight. These values are extremely low for normal persons.

Shaffer observed, as early as 1907, that there usually was a very small excretion of creatinine present in the urine of patients who were in bed, irrespective of the cause of their disease. According to Shaffer this small excretion is found with a considerable number of pathological persons under very varying circumstances. Further it was found that abnormally small excretion of creatinine is not at all specific for any disease whatever.

Shaffer fails to explain this phenomenon, because absence of muscular work cannot be of influence; and besides this the only other possible causes, that can be of any importance, are intoxication and a change of tonus in consequence of the disease.

There are objections, however, also against the two last mentioned factors, because it can hardly be possible that the persons, whose excretion of creatinine I have given, were so enormously intoxicated as to excrete only ± 6·5 mg. of creatinine instead of the usual ± 20 mg., whilst they showed no other obvious symptoms of intoxication. The muscular tonus of these patients was normal so that this factor could not be of much weight.

*Spastic cases.* Eight patients with increased muscular tonus were examined, and these had the following varying diseases: post-apoplectic paralysis, tuberculous spondylitis, operated benign tumor myeli, cerebro-spinal syphilis, trauma of the spinal column, spastic spinal paralysis and multiple sclerosis. In all the cases mentioned an increased amount of creatinine was found in comparison with the normal values given above. The figures obtained varied between 8·8 and 13·2 mg. per diem per kilo. of body-weight, and this gives an average of 11·5 mg. per kilo.

*Hypotonic cases.* On the strength of the tonus theory a lowering of the creatinine value could be expected; but on the contrary it was found that two patients with tabes dorsalis, one with multiple sclerosis, and one with hereditary ataxia (Friedreich) with hypotonic muscles, showed distinctly increased excretions, namely 9·7–14 mg. per kilo. of body-weight with a mean value of 11·4 mg.

How can all this be interpreted? It is plain that muscular tonus cannot be the only factor that is bearing on the excretion of creatinine, because not only do we find an increased excretion of creatinine with increased tonus, but also with decreased tonus.

It is possible that some other additional factor is involved. When the cases examined are mutually compared, it is striking to find that they all show one common feature, and that is that the conduction of impulses from the cerebrum to the muscles or *vice versa* is interrupted or in any case badly hampered through an injury to one or more of the long tracts.

An increased excretion of creatinine is therefore also to be expected in those nervous disorders in which the connexions between brain and muscles are damaged without appreciable changes in the tonus. I have, in fact, found in two cases of multiple sclerosis with increased reflexes, Babinski's reflex and ataxis, but without changes in the tonus, the values 10 and 15·4.

It appeared to me to be interesting to investigate whether a functional interruption of the tracts between the motor and sensory areas of the cortex and the muscles would influence the creatinine metabolism.

For this purpose the following two series of patients were examined:

(1) Patients with preoccupied consciousness.

(2) Patients with diffused consciousness.

Both in cases of intense preoccupation and diffused consciousness the normal peripheral innervation is inhibited. In illustration of this last point I wish to mention: obstipation with melancholia; catalepsy in cases of dementia praecox; disappearance of the arrhythmia in the pulse with preoccupation and an increase of these differences with patients of diffused consciousness, according to Wiersma [1913].

*Preoccupied consciousness.* The urine of seven cases of hysteria, of one patient with traumatic neurosis, and of five cases of melancholia, was examined, and the average excretions were 11·8, 15·6 and 12·1 respectively.

One case of psychasthenia gave an average of at least 15. Two cases of mania gave averages of 12·3 and 14·3 respectively. None of these patients showed any signs of increased muscular tonus. As a matter of fact in some of the cases of melancholia a *lowered tonus* was present and moreover this did not influence the excretion of creatinine.

*Diffused consciousness.* With two patients with cerebral tumours, who were dull and had hemipareses, the excretions of creatinine were 11·6 and 15·2; in two cases of dementia epileptica, 13·2 and 18·5; in two cases of dementia praecox, 15·7 and 16·9; in two cases of dementia paralytica, 18·2 and 14·5; in one case of dementia senilis, 12·7.

## Conclusions.

In comparison with normal persons, who were under external circumstances similar to those of the patients experimented on, an increased excretion of creatinine was found with patients suffering from nervous disorders. The disorders were of such a nature that the normal connexions between muscles and brain were damaged and further that the patients were preoccupied and diffusely conscious.

In contradistinction to Pekelharing and Van Hoogenhuyze an increased creatinine excretion was found also with lowered muscular tonus. The question now arises how this high excretion, that is generally accepted as the normal value, is to be explained? By far the greatest number of persons examined in the past were physiologists and students. These people naturally lived under very different psychical circumstances from those of the patients with whom they were compared. It is questionable whether, in the light of the above experiments, the higher values obtained with the students are not to be accounted for by the fact that these people are continuously preoccupied; whilst the patients in a hospital have a calm and regular life and do no or very little mental work.

To answer this question, I have, after the completion of the foregoing experiments, examined the urine of some other patients in the local hospital. The investigations included: a soldier with practically cured otitis media; a case of ischias; one of compensated mitral insufficiency; one of ulcus ventriculi; one of almost cured pleuritis tuberculosa and one of chronic bronchitis. All these cases can be considered more or less normal as far as their metabolism is concerned.

To my great astonishment they all gave high excretions, which correspond to the normal values given by former investigators. Unluckily I cannot as yet explain this phenomenon. It is however of importance, in connexion with the foregoing, to state that some of these last patients gave a distinctly high excretion of creatinine on a certain day, when they were in an emotional state of mind. This also points to the fact that the mind is of influence on the excretion of creatinine. I hope to investigate more closely the different nervous and psychical factors, that bear on the formation and excretion of creatine and creatinine, at an early date.

In any case we can accept, and this is in keeping also with what other observers found in cases of nervous disorders, that the muscular tonus is not the most important factor in the excretion of creatinine.

When the generally accepted normal values are used as standards of comparison, it is seen that there is, in cases of nervous diseases with both increased and lowered tonus, an equal or slightly less quantity of urinary creatinine. If we are justified, however, in taking as normal the values that I have found for normal persons in the first experiments, then it is clear that both increased and decreased tonus is accompanied by increased excretion. In this case it can also be stated that the functional interruption of the nervous connexions between cerebral cortex and muscles, in preoccupied and diffused consciousness, causes an increased excretion.

SUMMARY.

In summing up the above mentioned observations, the following conclusions are legitimate:

(1) The muscular tonus cannot be the preponderating factor in the excretion of creatinine.

(2) It is probable that the condition of the tracts between the cerebrum and the muscles influences the excretion of creatinine in the urine.

(3) An important influence on the excretion of creatinine in the urine should be assigned to the mind.

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# XXXIV. THE SALT EFFECT IN ALCOHOLIC FERMENTATION.

By ARTHUR HARDEN and FRANCIS ROBERT HENLEY.

*From the Biochemical Department, Lister Institute.*

(*Received March 15th, 1921.*)

When the necessary hydrogen acceptor (in the form of acetaldehyde) is supplied to a mixture of yeast juice and sugar containing the appropriate concentration of phosphate, the maximum rate of fermentation is attained with practically no delay [Harden and Henley, 1920]. If, however, the acetaldehyde be omitted, the other conditions remaining unchanged, there will be a considerable delay in attaining the maximum rate of fermentation. With increasing concentration of phosphate, the period of delay is lengthened, as the maximum rate of fermentation is in general not attained until the concentration of phosphate has been reduced approximately to the optimum by the formation of hexosephosphate (see Figs. 1 and 2). If the concentration of phosphate be raised still more a point is reached at which the rate of fermentation never reaches the maximum. In presence of added acceptor (acetaldehyde) the maximum rate of fermentation attainable and optimum concentration of phosphate are higher than in its absence [Harden and Henley, 1920].

It appears therefore that the enzymes concerned are extremely sensitive to even a slightly excessive concentration of phosphate.

Meyerhof [1918] has shown that NaCl and other salts exercise a similar depressing effect both on the rate of attainment of the maximum and on the maximum rate attained and he therefore considers that phosphate, in addition to its specific function, exerts a general effect on the process which is shared by other salts. The following experiments were undertaken with the object of comparing the relative effects of different salts and of ascertaining whether the effect is a general one or is specific to any particular stage of the fermentation process.

It was found in the first place, in full confirmation of Meyerhof's results, that the effect of other salts is in general similar to that of excess of phosphate. Using equimolecular solutions the chlorides of Na and K have about the same effect, which is, however, less than that of the corresponding sulphates.

As regards the effect in different stages of the process, direct experiment showed that chlorides and sulphates, in the concentrations employed in these experiments, had no depressing effect on the rate of decomposition of pyruvic acid, whilst phosphates had a slightly stimulating effect, probably due to the

diminution of [H$^+$] in their presence. It was next established that the characteristic salt effect was not removed or greatly modified by the addition of acetaldehyde. Finally it was found that the rate of action of the hexose-phosphatase was diminished by the salts employed.

As regards the effect of excess of phosphate it was found that this differs from that of sulphates and chlorides by being relatively greater in the absence of acetaldehyde and less in its presence.

*Experimental.*

I. Effect of NaCl, $Na_2SO_4$, KCl and $K_2SO_4$ on the fermentation of fructose and glucose by zymin.

Expt. 1. The fermenting mixtures had a concentration 0·1 *M* of $K_2HPO_4$ and 0·25 *M* of the experimental salt. The maxima attained and the times required for their attainment were as follows:

| | Maximum rate cc. per 5 mins. | Time mins. |
|---|---|---|
| Control | 7·7 | 35 |
| NaCl | 5·2 | 48 |
| $Na_2SO_4$ | 4·3 | 57 |
| KCl | 5·2 | 45 |
| $K_2SO_4$ | 4·4 | 52 |

The results with glucose and fructose were similar. Meyerhof's observations are thus confirmed and it is further to be noticed that the chlorides of Na and K have about the same effect, which is less than that produced by the corresponding sulphates. The greater effect of these latter salts indicates that it is probably the anion of the salt which is efficacious.

II. Effect of salts on particular stages of the fermentation process.

A. *Effect of* $K_2HPO_4$, KCl *and* $K_2SO_4$ *on the action of carboxylase.*

In order to obviate the difficulties introduced by autofermentation, washed zymin was used in presence of a pyruvate and boric acid [see Harden, 1913]. The pyruvate solution was made by neutralising 2·42 g. of pyruvic acid with KHO and making to 25 cc.

Expt. 2. Five flasks were made up each containing 10 cc. of washed zymin suspension (2 g. zymin), and 0·2 cc. toluene with the following additions, the total volume being 15 cc.

| No. | Glucose | Pyruvate | Boric acid | 1·2 *M* $K_2HPO_4$ | 1·2 *M* KCl | Water |
|---|---|---|---|---|---|---|
| 1 | 0·5 g. | 0 cc. | 0 g. | 0·75 cc. | 0·5 cc. | 13·5 cc. |
| 2 | 0 | 6 | 1·2 | 0 | 0 | 9 |
| 3 | 0 | 6 | 1·2 | 9 | 0 | 0 |
| 4 | 0 | 6 | 1·2 | 0 | 4 | 5 |
| 5 | 0 | 6 | 1·2 | 0 | 9 | 0 |

After incubation for 10 minutes at 25°, readings of the gas evolution were taken at frequent intervals, with the following results. The absence of fermentation in No. 1 shows that the washing had been efficacious.

| No. | Total gas evolved 1 hour | 2 hours | 4 hours |
|---|---|---|---|
| 1 | 0·1 cc. | 0·1 cc. | 0·1 cc. |
| 2 | 15·3 | 18·9 | 25·6 |
| 3 | 19·0 | 26·8 | 33·7 |
| 4 | 13·4 | 18·6 | 23·0 |
| 5 | 13·7 | 19·0 | 23·2 |

By comparing the results of Nos. 2, 4 and 5, it is seen that the KCl has had practically no effect, though present in higher concentration than in the experiment previously described (Expt. 1). A similar result was obtained on another occasion with $K_2SO_4$.

The $K_2HPO_4$ has had a slight beneficial effect as the total gas produced in No. 3 is greater than that in No. 2. This effect may perhaps be due to a lowering of the [H·] by the $K_2HPO_4$.

B. *Effect of* $K_2SO_4$ *and of acetaldehyde on the rate of hydrolysis of hexosephosphate by hexosephosphatase.*

In presence of excess of sugar the rate of fermentation produced by either yeast juice or zymin is conditioned by the concentration of free phosphate. As soon as any free phosphate present in the juice has been converted into hexosephosphate the rate of renewal of the supply depends mainly on the rate of action of the hexosephosphatase present. In these circumstances the rate of fermentation is a measure of the rate of action of the hexosephosphatase [see Harden, 1914, p. 119]. By adding a salt solution to a fermenting mixture in which these conditions have been attained, it is possible to determine whether the added salt has any effect on the hexosephosphatase rate.

A study of the effect of phosphate on the rate of action of hexosephosphatase cannot be made in this way because it enters into the reaction and the active masses of the reacting substances are thereby changed.

The effect of $K_2SO_4$ was tried both in the absence and presence of acetaldehyde for the sake of comparison with later experiments.

Expt. 3. Four flasks were made up each containing 2 g. zymin + 2 g. glucose + 0·2 cc. toluene and the following additions were made:

| No. | 1 % acetaldehyde | Water | 0·4 *M* $K_2SO_4$ | |
|---|---|---|---|---|
| 1 | 0 cc. | 20·9 cc. | 0 cc. | |
| 2 | 3 | 17·9 | 0 | |
| 3 | 0 | 13·4 | 7·5 | Added after the steady rate had been attained. |
| 4 | 3 | 10·4 | 7·5 | |

After a steady rate had been attained, readings were made every 5 minutes, with the following results:

| | | | | | | |
|---|---|---|---|---|---|---|
| No. 1 | Average rate over | 125 | minutes | 0·71 | cc. $CO_2$ per | 5 mins. |
| 2 | ,, ,, | ,, | ,, | 0·71 | ,, | |
| 3 | ,, ,, | 80 | ,, | 0·45 | ,, | |
| 4 | ,, ,, | ,, | ,, | 0·59 | ,, | |

The added $K_2SO_4$ has caused a marked reduction in the rate but this is not so great in the presence of acetaldehyde.

C. *Salt effect in presence and absence of added acceptor (acetaldehyde). Comparison of effects produced by* $K_2SO_4$ *and* $K_2HPO_4$.

Expt. 4. Six flasks were made up each containing 20 cc. of yeast-juice + 2 g. fructose + 0·2 cc. toluene, and the following additions were made:

| No. | 1 % acetaldehyde | Water | | 0·4 *M* $K_2SO_4$ | 0·6 *M* $K_2HPO_4$ | Water |
|---|---|---|---|---|---|---|
| 1 | 0 cc. | 5 cc. | | 0 cc. | 10 cc. | 7·5 cc. |
| 2 | 5 | 0 | And after | 0 | 10 | 7·5 |
| 3 | 0 | 5 | incubation | 7·5 | 10 | 0 |
| 4 | 5 | 0 | at 25° for | 7·5 | 10 | 0 |
| 5 | 0 | 5 | 25 mins. | 0 | 15 | 2·5 |
| 6 | 5 | 0 | | 0 | 15 | 2·5 |

Readings of the gas evolution were taken at intervals of 5 minutes.

As the concentration of phosphate in the fermenting liquid is progressively changing, whereas the concentration of $K_2SO_4$ presumably remains unchanged, it is useless to make a direct comparison of the rates of fermentation observed in the several experiments at equal intervals of time measured from the moment at which the various additions were made. Since a simple relation exists between the $K_2HPO_4$ added and the $CO_2$ evolved in excess of that which would have been produced had no $K_2HPO_4$ been added, it is possible to calculate from the total gas evolution, measured from the time at which additions were made, the amount of free phosphate remaining at any moment during the course of the experiment. When this calculation has been made at a series of intervals during each experiment, comparison of the various experiments can be made at equal concentrations of free phosphate. In making the calculations it is assumed that 1 cc. of 0·6 *M* $K_2HPO_4$ is equivalent to 14·8 cc. of $CO_2$ under the conditions of the experiment, and that 0·9 cc. $CO_2$ must be allowed as the normal rate of fermentation in absence of added phosphate. The latter assumption is not strictly accurate for all the experiments, since the normal rate of fermentation, *i.e.* the hexosephosphatase rate, is affected by the addition of phosphate or sulphate as shown in section B above. The errors introduced from this cause are probably not large, and are to some extent unavoidable, as it is very difficult to estimate the hexosephosphatase rate exactly after the addition of large amounts of phosphate. The curves shown in Figs. 1 and 2 have been prepared in this way by calculation from the observed gas evolution in experiment 4 (Nos. 1–6). In the cases (Nos. 5 and 6) in which the effect of 5 cc. of extra phosphate is to be determined, it is necessary to assume the available free phosphate as equivalent to only 10 cc. of the total of 15 cc. which are actually present, and only to utilise the observations made while the extra 5 cc. of phosphate remained intact. Hence the comparison can only be carried as far as the point at which the concentration of phosphate in the experiments with 10 cc. of phosphate alone, or in presence of $K_2SO_4$ (Nos. 1, 2, 3, 4) is such as to produce the maximum rate. After this point the rate in these experiments falls off for lack of phosphate, whereas in the experiments with the extra 5 cc. of phosphate (Nos. 5 and 6) this does not occur so soon, and the rate maintains a higher level at the expense of some of the extra phosphate.

In order to compare the depressing effects of the added salts the ratio of the rates of fermentation, at points on the curves where the available phosphate concentration is equal, are employed. These ratios may legitimately be calculated at any points of equal phosphate concentration. But it should be noted that the results at the beginning and end of the experiments are not so trustworthy as the others. In the former case, because the rates are changing rapidly, in the latter because with diminishing phosphate and rate of fermentation the relative errors of calculation are greatly increased.

The results of the observations in absence of added acetaldehyde are shown in Fig. 1, and in presence of acetaldehyde in Fig. 2.

When the curves 1 and 3 ($K_2SO_4$ in absence of acetaldehyde) and 2 and 4 ($K_2SO_4$ in presence of acetaldehyde) are compared it is found that the ratios are fairly constant over a considerable range of free phosphate concentration, the values being given in the following table.

*Depressing effect of* $K_2SO_4$.

| Available phosphate in cc. of $CO_2$ | Without acetaldehyde Ratio $\frac{\text{Rate from 3}}{\text{Rate from 1}}$ | With acetaldehyde Ratio $\frac{\text{Rate from 4}}{\text{Rate from 2}}$ |
|---|---|---|
| 135 | 0·78 | 0·77 |
| 130 | 0·78 | 0·78 |
| 120 | 0·77 | 0·77 |
| 110 | 0·76 | 0·77 |
| 100 | 0·74 | 0·76 |
| 90 | 0·71 | 0·75 |
| Average ... ... | 0·76 | 0·77 |
| % Depression ... | 24 | 23 |

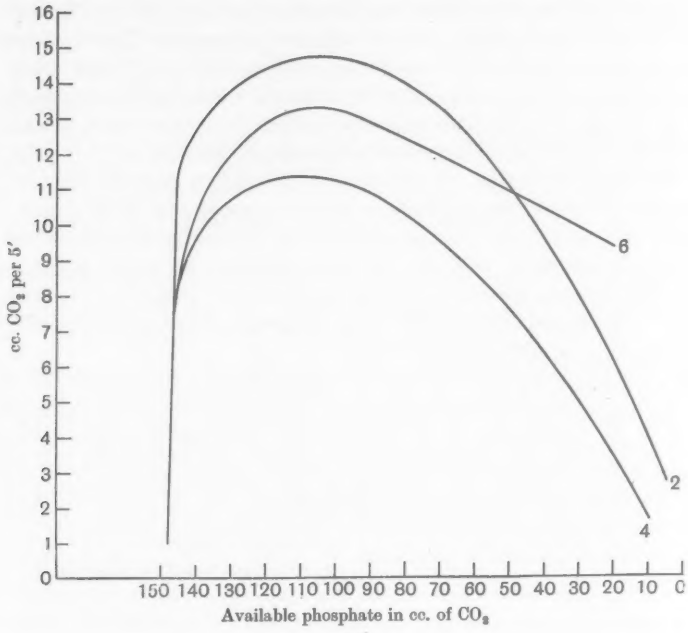
Hence the depressing effect of $K_2SO_4$ is the same in presence or absence of acetaldehyde.

When, however, the ratios are calculated from 1 and 5 (extra phosphate in absence of acetaldehyde) and from 2 and 6 (extra phosphate in presence of acetaldehyde) it is found that they are very different in the two cases.

*Depressing effect of phosphate.*

| Available phosphate in cc. of $CO_2$ | Without acetaldehyde Ratio $\frac{\text{Rate from 5}}{\text{Rate from 1}}$ | With acetaldehyde Ratio $\frac{\text{Rate from 6}}{\text{Rate from 2}}$ |
|---|---|---|
| 130 | 0·58 | 0·85 |
| 120 | 0·60 | 0·88 |
| 110 | 0·61 | 0·89 |
| 100 | 0·60 | 0·89 |
| 90 | 0·58 | — |
| Average ... ... | 0·59 | 0·88 |
| % Depression ... | 41 | 12 |

It is seen from these figures that the depression produced by extra phosphate is greatly diminished by the presence of acetaldehyde and it would hence appear that a large part of the depressing effect of phosphate is exercised on the production (or possibly the reduction) of the acceptor which is necessary for the attainment of a high phosphate rate [see Harden and Henley, 1920, 1921].


16
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cc. $CO_2$ per 5'
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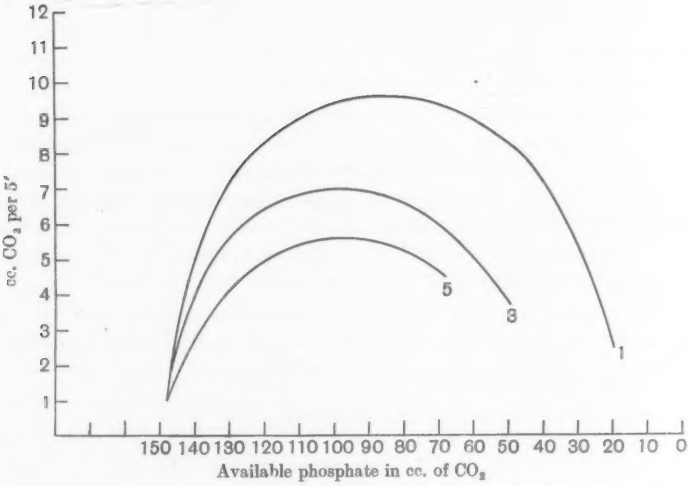
150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
Available phosphate in cc. of $CO_2$

Fig. 1.


12
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1
cc. $CO_2$ per 5'
5
3
1
150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
Available phosphate in cc. of $CO_2$

Fig. 2.

The sulphate on the other hand does not appear to have this specific action, since the effect is not altered by the addition of acceptor. The effect which it does exercise, and which may be regarded as the general salt effect of Meyerhof, appears to be a depression of the rate of one or more of the reactions in an early stage of the fermentation process, since, as we have seen, this salt is without any marked action on the decomposition of pyruvic acid by carboxylase. Whether the residual effect of phosphate, which is exercised in presence of acetaldehyde, is of the same nature or not cannot at present be stated [see Harden, 1914, p. 72]. It is interesting to note that whilst the total effect of phosphate in absence of acetaldehyde is much greater than that of sulphate, the residual effect in presence of acetaldehyde is much less. It is also worthy of note in this connexion that the normal function of phosphate is probably connected with one of these early stages.

Parallel experiments with glucose and yeast juice in which the concentrations of phosphate and sulphate were only half as great as in the experiments with fructose gave results in good agreement with those described above. The percentage depressions produced by $K_2SO_4$ were: in presence of acetaldehyde, 23 %; in absence of acetaldehyde, 26 %. The percentage depressions produced by $K_2HPO_4$ were: in presence of acetaldehyde, 17 %; in absence of acetaldehyde, 41 %. Since the ratio $\frac{\text{concentration of } K_2HPO_4}{\text{concentration of } K_2SO_4}$ is the same in both series of experiments and since the percentage depressions are approximately equal, it appears to follow that these salts affect the fermentation of glucose by yeast juice in a similar manner to that of fructose.

The salt effect seems to be correlated with the phosphate rate; and just as the maximum phosphate rate and tolerance of phosphate are greater with fructose than with glucose the tolerance of salt is also greater in the case of fructose.

## Summary.

1. As stated by Meyerhof, the chlorides and sulphates of sodium and potassium exert a depressing effect on the maximum rate obtainable and the rate of attainment of this maximum in the fermentation of glucose and fructose by yeast juice or zymin in the presence of phosphate. The effect of the sulphates is greater than that of the chlorides.

2. Salts diminish the rate of action of hexosephosphatase, but are without effect on that of carboxylase.

3. The depressing effects produced by potassium sulphate and by excess of phosphate differ in character, the latter being greatly diminished by addition of acetaldehyde, whereas the former is practically unaffected.

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# XXXV. OBSERVATIONS ON ABNORMAL IODINE VALUES, WITH SPECIAL REFERENCE TO THE STEROLS AND RESINS.

By IDA SMEDLEY MacLEAN and ETHEL MARY THOMAS.

(*Received March 16th, 1921.*)

While investigating the nature of yeast fat [Smedley MacLean and Thomas, 1920] we noticed that the sterol of yeast fat exhibits a marked difference in its iodine value when this is determined respectively by the Wijs and Hübl methods. This abnormal behaviour was described by Werner [1911] for cholesterol and sitosterol and is referred to in the text-book of Lewkowitsch [1913]. It seemed to us desirable to try and ascertain how far this property was peculiar to the sterols and whether it could be shown to be due to any definite grouping of atoms.

The iodine value of cholesterol was first determined by Lewkowitsch [1892] by Hübl's method; he obtained the number 65·8, agreeing approximately with that required by the presence of one double bond ($C_{27}H_{46}O.H_2O$ requires 62·8). Using the same method, Werner found for sitosterol values ranging from 41 to 76 but after only half an hour's contact with the Wijs solution the iodine number had reached 135.

The sterols examined in the present investigation were cholesterol, brassicasterol, yeast sterol (ergosterol), dihydrocholesterol and coprosterol; of these the last two alone show only slight absorption of iodine with either the Wijs or Hübl solution.

*Resins* also show great variations in iodine values according to the conditions of the determination, the Wijs values reaching higher figures than the Hübl ones. McIlhiney [1894, 1902] showed that the Hübl iodine number of the resins was considerably affected by the time of absorption and by the excess of halogen used. Smetham and Dodd [1900] found that the Wijs numbers were similarly influenced. American colophony and the abietic acid obtained from it also give abnormally high Wijs values and will be discussed later.

*The naphthenic acids* constitute another class of substances which are described as showing differences in the iodine values determined respectively by the Hübl and Wijs methods; the iodine values are, however, low. The material used consisted of residues from natural petroleum and there is insufficient evidence as to the nature of the individual substances contained in them. Marcussohn [1905, 1907] and others have used the similar behaviour

of the naphthenic acids and cholesterol when treated with iodine reagents as an argument for the animal origin of petroleum, these acids being regarded as derived from cholesterol.

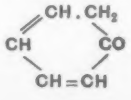
Wijs' reagent consists essentially of a solution of iodine monochloride in glacial acetic acid; Hübl's reagent is made by mixing solutions of mercuric chloride and iodine, each in 95 % spirit, and in this iodine monochloride is considered to be the active substance. We have, therefore, a solution in the one case of iodine monochloride in alcohol and in the other of the same compound in acetic acid; with those unsaturated compounds, in which addition of halogen is the only reaction, both appear to act similarly as iodinating agents.

A survey of the literature shows that the monochloride of iodine has been used before as an iodinating agent. Michael and Norton [1878] used this for the preparation of mono-, di- and tri-derivatives of phenol, aniline, and para-acetanilide, and Willgerodt [1901] for the iodination of the nitranilines, the degree of the iodination increasing with the relative amount of iodine chloride used. Sernow [1903] has also used this reagent for the iodination of acid chlorides.

Iodine monochloride, therefore, especially in acetic acid solution produces substitution in certain aromatic compounds and one product of this reaction is an iodo-derivative, whilst hydrochloric acid must also be formed. The presence of the acetic acid in the Wijs solution favours substitution. It is possible that the mercuric salt in the Hübl reagent may have a special influence in diminishing substitution.

The sterols and resin acids show in a well-marked degree the property of discriminating between the action of these two iodine reagents; we also examined a considerable number of substances of known structure in order to determine whether any special atomic groupings could be connected with this property. The iodine values determined by the Hübl and the Wijs methods respectively are shown in the accompanying table and the results are discussed below. Where substitution occurs, the iodine numbers vary within wide limits, depending on the time of contact and the proportions of the reacting substances, so that for such substances, there is no one definite iodine value.

A survey of the figures on pp. 321, 322 shows that the Hübl values are markedly greater than those obtained by the Wijs method in the case of phenol and to a much less extent in the case of naphthol and the cinnamyl series, alcohol, aldehyde and acid. The iodine values of the phenols were investigated first by Fahrion [1901]. The Hübl value is much higher than that determined by the Wijs reagent; the number of halogen atoms absorbed in the two cases being respectively 3·2 and 2·0. Wake and Ingle [1908] suggest that phenol reacts as a cyclic ketone



| | Formula | Hübl iodine value | Wijs iodine value | No. I atoms added by 1 mol. substance: Hübl | Wijs |
|---|---|---|---|---|---|
| **Hydrocarbons:** | | | | | |
| Benzene ... ... ... | $C_6H_6$ | 0 | 0 | 0 | 0 |
| Naphthalene ... ... | $C_{10}H_8$ | 0 | 0 | 0 | 0 |
| Fluorene ... ... ... | $C_{13}H_{10}$ | 5·7<br>6·2 | 18·2<br>20·5 | 0·1 | 0·2 |
| Anthracene ... ... | $C_{14}H_{10}$ | 171 to 237 | 149 to 256 | to 3·5 | to 3·8 |
| Phenanthrene ... ... | $C_{14}H_{10}$ | 10·8<br>11·5 | From 20·0[1]<br>to 100·9 | 0·1 | 0·2<br>to 1·3 |
| Retene ... ... ... | $C_{18}H_{18}$ | 13·4<br>14·4 | 116·1<br>115·0 | 0·2 | 2·1 |
| (1-methyl-7-*iso*propylphenanthrene) | | | | | |
| **Alcohols:** | | | | | |
| Normal primary alcohols (ethyl to octyl) | | 0 | 0 | 0 | 0 |
| Allyl alcohol ... ... | $C_3H_6O$ | 414·3<br>425·5 | 435·0<br>444·1 | 1·95 | 2·03 |
| $CH_2:CH.CH_2OH$ | | | | | |
| Cinnamyl alcohol ... | $C_9H_{10}O$ | 18·2<br>18·0 | 7·6<br>9·2 | 0·2 | 0·1 |
| $C_6H_5.CH:CH.CH_2OH$ | | | | | |
| Terpene alcohols ... | $C_{10}H_{18}O$ | 263·6 | 244·3[2] | — | 3·0 |
| Geraniol (B.P. 125–8°, 20 mm.) $(CH_3)_2C:CH.CH_2.CH_2.C(CH_3):CH.CH_2OH$ | | 264·0 | 277·4[3] | 3·2 | 3·4 |
| Linalool (B.P. 124°, 60 mm.) $(CH_3)_2.C:CH.CH_2.CH_2.C.(CH_3)(OH).CH:CH_2$ | | 367·8<br>374·0 | 217·6<br>216·0 | 4·5 | 2·6 |
| Terpineol ... ... ... | | 182·3 | 182·7 | 2·2 | 2·2 |
| Dihydrocarveol ... ... | | 174·6<br>180·8 | 182·3<br>184·3 | 2·2 | 2·2 |
| Menthol ... ... | | 0 | 0 | 0 | 0 |
| **Phenols:** | | | | | |
| Phenol ... ... ... | $C_6H_5OH$ | 473·3<br>484·8 | 274·8<br>274·6 | 3·5 | 2·0 |
| α-Naphthol ... ... | $C_{10}H_7OH$ | 277·9 | 274·6 | 3·2 | 3·1 |
| β-Naphthol ... ... | $C_{10}H_7OH$ | 177·9<br>184·0 | 174·3<br>183·1 | 2·1 | 2·1 |

[1] The values obtained varied from 26 to 101, the number increasing especially with the proportion and concentration of the iodine solution and with the temperature.

[2] With 15 cc. Wijs solution.

[3] With 30 cc. Wijs solution.

| | Formula | Hübl iodine value | Wijs iodine value | No. I atoms added by 1 mol. substance: Hübl | No. I atoms added by 1 mol. substance: Wijs |
|---|---|---|---|---|---|
| **ALDEHYDES:** | | | | | |
| Butyraldehyde ... ... | $C_4H_8O$ | 0 | up to 339 | 0 | 2·0 |
| *Iso*valeraldehyde ... ... | $C_5H_{10}O$ | 26·56<br>29·12 | 263·9<br>263·1 | 0·2 | 1·8 |
| Oenanthol ... ... | $C_7H_{14}O$ | 22·1<br>24·1 | 198·5<br>198·9 | 0·2 | 1·8 |
| Benzaldehyde ... ... | $C_7H_6O$ | 1·3 | 1·4 | 0 | 0 |
| Crotonaldehyde ... ... | $C_4H_6O$ | 334·3 | 173·6 | | |
| $CH_3.CH:CH.CHO$ | | 334·2 | 171·8 | 1·9 | 1·0 |
| Citral ... ... ... | $C_{10}H_{16}O$ | 268·1 | 268·4 | | |
| $CH_3.C(CH_3)=CH.CH_2.CH_2.C(CH_3)=CH.CHO$ | | 281·0 | 287·8 | 3·4 | 3·4 |
| Cinnamaldehyde ... ... | $C_9H_8O$ | 143·3 | 85 | 1·5 | 0·9 |
| $C_6H_5CH:CH.CHO$ | | 156·7 | 81 | | |
| **KETONES:** | | | | | |
| Acetone ... ... ... | $CH_3CO.CH_3$ | 21·1<br>24·1 | 164·6<br>165·0 | 0·1 | 0·8 |
| Acetophenone ... ... | $C_6H_5.CO.CH_3$ | 0 | 204·2[1] | 0 | 1·9 |
| **QUINONES:** | | | | | |
| Anthraquinone ... ... | | 0 | 0 | 0 | 0 |
| Phenanthraquinone | | 0 | 0 | 0 | 0 |
| **STEROLS:** | | | | | |
| Cholesterol ... ... | $C_{27}H_{45}O.H_2O$ | 72·8<br>72·1 | 145·7[2]<br>164·3 | 2·3 | 5·0 |
| Dihydrocholesterol ... | $C_{27}H_{48}O.H_2O$ | 2·6 | 3·0<br>2·6 | 0·1 | 0·1 |
| Coprosterol ... ... | $C_{27}H_{48}O$ | 23·8[3]<br>9·3[4] | 33·2[3]<br>18·2[4] | 0·7<br>0·3 | 1·0<br>0·55 |
| Brassicasterol ... ... | $C_{28}H_{46}O.H_2O$ | 118·6 | 282·2 | 3·9 | 9·2 |
| Yeast sterol (ergosterol) ... | $C_{27}H_{42}O.H_2O$ | 207·2<br>177·1 | 339·5<br>337·8 | 6·5<br>5·6 | —<br>10·7 |
| **ACIDS:** | | | | | |
| Crotonic ... ... ... | $CH_3CH:CH.COOH$ | 25·0[5]<br>25·9 | — | ·09 | — |
| Cinnamic ... ... ... | $C_6H_5CH:CH.COOH$ | 12·75<br>4·7[5] | 6·2 | ·08 | ·03 |
| Abietic ... ... ... | $C_{20}H_{30}O_2$ | 156·6<br>162·1 | 239·4<br>242·5 | 3·8 | 5·7 |
| Dihydroabietic ... ... | $C_{20}H_{32}O_2$ | 75·1 | 237·4<br>231·8 | 1·8 | 5·6 |

[1] See p. 323.
[2] Values vary with iodine concentration and temperature, see p. 326.
[3] Left for 24 hours at laboratory temperature in summer.
[4] In winter.
[5] Lewkowitsch [1913].

It will be shown later that the Wijs reagent favours the change from the ketonic to the enolic form and it is noteworthy that where the reverse change may be involved Hübl's solution gives the larger numbers.

The naphthols show little difference between the actions of the two iodine reagents, but the number of halogen atoms absorbed is greater with $\alpha$-naphthol than with the $\beta$-compound. If the halogen absorption is conditioned by a

preliminary change from the enolic to the ketonic form, it would follow that (1) the Wijs reagent has a more potent influence in maintaining the enolic form of phenol than that of the naphthols and (2) that $\alpha$-naphthol reacts more readily as a cyclic ketone than does the $\beta$-compound.

The abnormalities shown by the terpene alcohols may be explained if two facts are taken into account: (1) the nature of the groups attached to the double bonds and (2) the action of the acetic acid in the Wijs solution. In geraniol the double bond is in the $\alpha$, $\beta$-position to the primary alcohol group, and the effect of the two alkyl groups attached to the $\beta$ carbon and of the $CH_2OH$ group attached to the $\alpha$ carbon atom is to diminish the iodine absorption, just as in cinnamyl alcohol the position of the phenyl and $CH_2OH$ groups prevents the iodine absorption (cp. p. 324). In geraniol, therefore, both Hübl and Wijs values are low and, as might be expected, they are considerably influenced by the concentration of iodine. With linalool the Hübl value is slightly greater than normal, the Wijs value low. Zeitschel [1906] showed, however, that the effect of glacial acetic acid on linalool in the presence of certain chlorides was to convert linalool into a mixture of geraniol and nerol. The conditions to produce isomerisation of the linalool are present in the Wijs solution and may account for the low Wijs values obtained.

In certain cases, the Wijs values are somewhat higher than those obtained with the Hübl reagent, as for instance in the condensed ring hydrocarbons, naphthalene, fluorene and anthracene, but here the differences are small, corresponding to the absorption of from 0·1 to 0·2 of an iodine atom. It is possible also that some of these slight differences may be due to the presence of small amounts of impurities in the substances examined.

Four groups of substances however discriminate sharply between these two reagents giving much higher Wijs values.

(1) Carbonyl compounds (fatty aldehydes and ketones).

(2) Hydrocarbons containing the phenanthrene nucleus.

(3) The sterols with the exception of the reduced sterols, coprosterol and dihydrocholesterol.

(4) The resinic acids, abietic acid and to a greater extent its reduced derivative, dihydroabietic acid.

### (1) *Carbonyl compounds.*

With a considerable excess of Wijs reagent, the lower fatty aldehydes give iodine numbers corresponding approximately to the absorption of two iodine atoms by one molecule of the substance, the Hübl values being much lower. The proportion of iodine absorbed from Wijs solution appears to be greatly affected by the relative concentration of aldehyde and iodine: the blue starch iodine colour returns very rapidly after the end-point is reached. Acetone and acetophenone also discriminate between the two reagents.

The Hübl value of acetophenone was found to be nil—the Wijs value anything from 10 to 202, the latter number corresponding to the absorption

of two halogen atoms. The great variation in the Wijs values obtained was due to differences in the laboratory temperature.

Thus at − 10° the iodine value was 11·1; at 13° 151·9; at 26° 204·2; and at 36° 199·4. In an experiment left for the same time over night in the laboratory a value of only 34·2 was found. The night temperature was probably lower than 13° though the day temperature was 20°. Probably a temperature of over 20° is necessary to ensure that complete enolisation is produced by the Wijs solution.

Lapworth [1904] in his work on the halogenation of the ketones, showed that the addition of halogen to the carbonyl group is influenced by the presence of acids; their effect is to catalyse the change from the ketonic to the enolic form which was shown by Lapworth to precede addition. It appeared possible therefore that the glacial acetic acid used as the solvent for the iodine monochloride in the Wijs solution might help the enolisation of the ketone or aldehyde and so determine the absorption of iodine. We tried to test this by adding an equal volume of glacial acetic acid to the Hübl reagent, but under these conditions much of the acid is converted to ethyl acetate by the alcohol of the Hübl solution. The addition of an equal volume of concentrated hydrochloric acid (sp. gr. 1·16) to the Hübl reagent produced a great increase in the iodine value, but the blue starch-iodine colour returned so rapidly that the end-point was not very satisfactory.

| Name | Wijs iodine value | Hübl iodine value | Hübl and HCl iodine value |
|---|---|---|---|
| Butyraldehyde ... | 339 | 17·9 | 192·6 |
| Oenanthol ... | 183·0 | 40·4 | 125·7 |

The behaviour of benzaldehyde is in accordance with this explanation. Here, there is no possibility of enolisation and no iodine is absorbed. It seems probable therefore that the Wijs reagent acts by increasing the enolisation of the aldehyde or ketone and that the amount of halogenation depends on the degree of enolisation. It may well be that the glacial acetic acid used as solvent influences the enolisation.

The $\alpha, \beta$-unsaturated aldehydes show an entirely different behaviour. In the crotonic and cinnamyl series (alcohol, aldehyde and acid) the Hübl values are considerably greater than those obtained with the Wijs reagent; cinnamyl alcohol and cinnamic and crotonic acids give very small values with both reagents but the corresponding aldehydes absorb approximately two iodine atoms when Hübl's reagent is used. In the following table, the figures represent the number of iodine atoms absorbed per molecule of substances.

| Substance | Hübl | Wijs | Substance | Hübl | Wijs |
|---|---|---|---|---|---|
| Allyl alcohol ... $CH_2:CH.CH_2OH$ | 1·9 | 2·0 | Cinnamyl alcohol ... $C_6H_5CH:CH.CH_2OH$ | 0·2 | 0·1 |
| Crotonaldehyde ... $CH_3CH:CH.CHO$ | 2·0 | 1·0 | Cinnamyl aldehyde $C_6H_5CH:CH.CHO$ | 1·3<br>1·5 | ·85<br>·88 |
| Crotonic acid ... $CH_3CH:CH.COOH$ | 0·1 | — | Cinnamic acid ... $C_6H_5CH:CH.COOH$ | 0·1 | ·05 |

If the assumption made in the case of the saturated carbonyl compounds

is correct, namely that the enolisation of these compounds is increased by the acid of the Wijs solution, we must infer that in the conjugated aldehydes there is little tendency to enolisation. The greater protection against halogen addition afforded by the alcohol and carboxyl groups suggests that the aldehyde group when conjugated with an ethenoid group is itself concerned in the addition of halogen, which probably takes place in the following manner:

$$CH_3 . CH : CH . CH : O + X_2 \rightarrow CH_3 . CHX . CH : CH . OX \rightarrow CH_3 . CHX . CHX . CHO$$

The product formed by the action of Hübl solution on crotonaldehyde is an aldehyde; it contains iodine but very little chlorine and attacks the eyes. We have not yet succeeded in isolating it in a sufficiently pure condition for analysis.

The absorption of iodine by the unsaturated aldehydes is a gradual process, as will be seen from the following figures:

| | | | | | | | | Theory for one double bond |
|---|---|---|---|---|---|---|---|---|
| No. of hours ... ... | 2 | 4 | 6 | 18 | 24 | 48 | 168 | — |
| Iodine value of | | | | | | | | |
| (*a*) Crotonaldehyde ... | 44 | 66 | 107 | 140 | 172 | 174 | 202 | 350 |
| (*b*) Cinnamaldehyde... | 39 | 52 | 64 | 84 | 96 | 117 | 103 | 190·5 |
| (*c*) Citral ... ... | 169 | 241 | 257 | 283 | 304 | 374 | — | 167 |

In citral, the 5, 6 double bond seems to react normally: the conjugated carbonyl and 1, 2 ethenoid linkages show a gradual absorption similar to that of croton and cinnamic aldehydes. After deducting the value for two iodine atoms absorbed by the 5, 6 double bond, 1·4 iodine atoms were absorbed from Wijs solution in 24 hours.

The behaviour of cinnamic acid, the Hübl value of which was determined by Lewkowitsch [1913], is in accordance with Bauer's rule [1904] for the addition of bromine to unsaturated substances, *i.e.* the capability of the ethylene linkage to add on bromine is diminished by the attachment of one of the negative groups COOH, COOR, $C_6H_5$ or Br to both carbon atoms. The $C_6H_5$ and $CH_2OH$ groups (cinnamyl alcohol) or the $CH_3$ and COOH groups (crotonic acid) when attached to the two ethylene carbon atoms appear to be sufficient almost entirely to prevent formation of the iodochloride, although they do not prevent bromination.

The iodine values of the unsaturated ketones were determined by Ingle [1904]; his numbers are given below.

| Substance | No. I atoms absorbed by 1 mol. substance | |
|---|---|---|
| | Hübl | Wijs |
| Benzylidene-acetone (Ingle) ... ... ... ... $C_6H_5CH : CH . CO . CH_3$ | 2 | — |
| Benzylidene-acetophenone (Ingle) ... ... ... $C_6H_5CH : CH . CO . C_6H_5$ | 0·9 | 1·16<br>4·2 |
| Dibenzylidene-acetone (Ingle)... ... ... ... $C_6H_5CH : CH . CO . CH : CH . C_6H_5$ | 2·0 | — |
| Benzal-cinnamylidene-acetone (Ingle) ... ... $C_6H_5CH : CH . CH : CH . CO . CH : CH . C_6H_5$ | 4·0 | — |
| Dicinnamylidene-acetone (Ingle) ... ... ... $C_6H_5CH : CH . CH : CH . CO . CH : CH . CH : CH . C_6H_5$ | 6·0 | — |

Just as in the case of the unsaturated conjugated aldehydes, the values found are always below those required by the number of double bonds present in the molecule.

As we had at our disposal specimens of tetraphenylallene, tetraphenylethylene and diphenylbutadiene, we thought it would be of some interest to examine the effect of the conjugated ethenoid linkages and to compare the action of these unsaturated hydrocarbons when treated with Wijs reagent.

| Substance and formula | No. I atoms absorbed by 1 mol. substance (Wijs) | Observer |
|---|---|---|
| Styrolene ... ... ... ... ... $C_6H_5CH:CH_2$ | 2 | Ingle |
| Stilbene ... ... ... ... ... $C_6H_5.HC:CH.C_6H_5$ | 2 | ,, |
| Tetraphenylethylene ... ... ... ... $(C_6H_5)_2C:C(C_6H_5)_2$ | 0 | MacLean and Thomas |
| Tetraphenylallene ... ... ... ... $(C_6H_5)_2C:C:C(C_6H_5)_2$ | 2 | ,, |
| Diphenylbutadiene ... ... ... ... $C_6H_5.CH:CH.CH:CH.C_6H_5$ | 4 | ,, |
| Diphenyloctatetrene ... ... ... ... $C_6H_5CH:CH.CH:CH.CH:CH.CH:CHC_6H_5$ | 6 | ,, |

It is perhaps worthy of note that the allene structure even when each allene terminal carbon atom is attached to two phenyl groups adds on two halogen atoms.

### (2) *Hydrocarbons containing the phenanthrene nucleus.*

Phenanthrene and its 1-methyl-7-*iso*propyl derivative, retene, show a marked difference between the action of the two iodine reagents, the Wijs values being considerably higher than the Hübl numbers in both cases. The Wijs value was extremely variable, increasing with the excess of iodine used and with rise of temperature. Numbers lying between 26 and 101 were obtained. Anthracene differs from phenanthrene in that it absorbs halogen from both the Hübl and Wijs solutions to an almost equal extent. The only reduced phenanthrene derivatives we had an opportunity of studying were abietic and dihydroabietic acids. Although the presence of the carboxyl group introduces a new factor, the difference in behaviour of the reduced and unreduced acids suggests that the partially reduced phenanthrene nucleus exercises an important influence.

### (3) *The sterols.*

The Hübl iodine values of the sterols cholesterol, brassicasterol and ergosterol correspond approximately with the absorption required by the presence of one, two or three double bonds respectively in their molecules. This is in agreement with what is known of the constitution of these substances. The Wijs values vary within wide limits but may be from two to three times as much as those obtained by Hübl's method.

*Factors influencing the iodine values of the sterols.*

Our experience shows that when the sterols are treated with iodine reagents, the iodine value obtained is largely influenced by (i) the time during which the sterol is in contact with the iodine solution; (ii) the relative concentration of iodine and sterol and (iii) the temperature of the reaction. The Hübl values are much less affected by these conditions than are the Wijs values and the former are always considerably lower than the latter.

(i) *Influence of the time of reaction.*

The iodine value determined after leaving 0·1 g. of cholesterol in contact with 15 cc. of the reagent for varying periods of time is shown in the following table:

| No. of hours | Hübl | Wijs |
|---|---|---|
| 6 | 58·65 | 141·6 |
| 18 | 70·2 | 152·7 |
| 24 | 73·2 | 153·7 |
| 48 | 71·8 | 165·4 |

(Theory for one double bond in cholesterol $C_{27}H_{46}O.H_2O$ requires iodine value 62·8.)

After 18 hours' contact, the Hübl value is practically stationary; the Wijs number increases somewhat on further standing.

(ii) *Relative concentration of iodine and sterol.*

Quantities, each of 0·1 g. of cholesterol, were left for 24 hours with varying amounts of iodine solution; the results were as follows:

| Weight of cholesterol | No. of cc. of iodine reagent | Hübl iodine value | Wijs iodine value |
|---|---|---|---|
| 0·1 g. | 10 | 71·45 | 103·6 |
| „ | 15 | 72·8 | 132·9 |
| „ | 20 | 73·2 | 164·3 |
| „ | 25 | 72·1 | 172·6 |
| „ | 30 | 73·3 | 180·5 |

(Theory for one double bond 62·8.)

The absorption from Wijs solution is markedly influenced therefore by increasing the relative proportion of the reagent; with Hübl's solution, on the other hand, this factor is almost without effect.

(iii) *Influence of temperature.*

When the iodine value of cholesterol was determined by the Wijs method, the same proportions of sterol and Wijs solution being left in contact for 24 hours, there were still wide variations in the values obtained, and we conclude that the day's temperature must exert a marked influence. The effect of increasing the temperature is shown in the following table:

| | Temperature | Hübl iodine value | Wijs iodine value |
|---|---|---|---|
| (Cold room) ... ... | 0° | 72·8 | 106·2 |
| (Antechamber to cold room) | 10°–13° | 74·7 | 115 |
| (Laboratory) ... ... | Between 15° and 20° | 74·6 | 128 |
| (Incubator) | 26° | 76·5 | 144 |
| (Hot room) | 36° | 74·5 | 174 |

0·1 g. of cholesterol was treated with 15 cc. of the iodine solution for 24 hours.

At 36° the Hübl control showed a considerable diminution, the amount of available halogen in it having decreased. The increase with rise of temperature is again very much larger in the case of the Wijs than of the Hübl solution.

With Hübl's solution the values obtained were always slightly greater than those required by the presence of one double bond in the molecule. The highest value obtained corresponded to the absorption of $1\frac{1}{3}$ molecules of halogen. As long as the sterol and iodine solution were left together for 24 hours, the result was very little influenced by the relative concentrations of iodine reagent and sterol. It was somewhat more influenced by a rise in the external temperature.

With the Wijs reagent on the other hand, the iodine values are markedly affected both by an increase in the external temperature and by an increase in the relative amount of the Wijs reagent. The Wijs numbers are from two to three times as great as the Hübl values. The numbers obtained in an ordinary laboratory determination vary markedly, being affected both by the temperature of the day and the proportion of sterol to available halogen in the Wijs solution.

A specimen of dihydrocholesterol, originally obtained by Willstätter and Mayer [1908] by reducing cholesterol with hydrogen in the presence of platinum black, melted at 139°–140° (uncorrected) and showed a specific rotation of + 21°. From neither iodine reagent was an appreciable amount of iodine absorbed. A specimen of coprosterol, for which our thanks are due to Mr J. A. Gardner, showed a low iodine value of the same order with both reagents. As the number obtained corresponded to the absorption of less than one atom of iodine, it may have been due to some small amount of impurity in the specimen, or it may be conditioned by the substance itself.

The difference in the behaviour of the two reagents when reacting with the unreduced sterols is therefore associated with the presence of the double bond in the reduced nucleus.

It is difficult to explain the very high values obtained with the Wijs solution in the cases enumerated otherwise than by assuming that a large amount of substitution takes place. The use of iodine monochloride in glacial acetic solution as the halogenating agent, and the presence in the structure to be attacked of a reduced nucleus of condensed rings in which one double bond is present, appear specially favourable conditions.

### (4) *The resins—abietic acid.*

Abnormal Wijs iodine values appear to be characteristic of the resin acids. The chief constituent of American colophony, known as abietic acid, has a much higher iodine value when treated with Wijs' than with Hübl's reagent. It is described as crystallising in colourless triangular plates, which are easily oxidised by the action of air, much more readily if kept in solution. Since

there are scarcely two observers who agree in the values they assign to its rotation and melting point, it is believed to be a mixture of two or more isomorphous acids. Rotations from + 66° [Henry, 1901] to − 66° [Mach, 1894] have been assigned to it, whilst the melting points ascribed vary from 120° [Flückiger, 1887] to 198° [Klason and Köhler, 1906].

Pimaric acid, isolated from French resin (galipot), melting at 211°–212° and having a specific rotation of + 75·5°, is considered by Easterfield and Bagley [1904] as the next homologue of abietic acid.

The resinic acids cannot yet be said to have been satisfactorily separated into individual substances of undoubted identity. The difficulty is increased since resins of different origins apparently vary in their constituents. Klason and Köhler [1906] succeeded in isolating from certain Scandinavian resins two acids, one melting at 168°–173°, $[\alpha_D] = + 52°$, and one melting at 198°, $[\alpha_D] = - 60°$. These acids closely resemble each other in chemical properties and it seems probable that what has been described as abietic acid is a mixture of these or of closely related acids.

We tried various methods described for the preparation of abietic acid and found most satisfactory the repeated distillation of the resin at 20 mm. pressure, with subsequent recrystallisations from methyl and ethyl alcohols [Easterfield and Bagley, 1904; Levy, 1905]. We found that if the recrystallisations are carried out at 0°, it is easier to obtain a specimen free from a yellow tinge. We did not, however, succeed in raising our melting point to 182° as described by Levy. Working in this way with two different specimens of resin, both sold to us as American colophony, we obtained from one an acid which softened at 155° and melted at 162°, $[\alpha_D] = - 73·5°$, and from another an acid melting at 166° after softening at 154°, $[\alpha_D] = + 37°$. On examination, the original resins were found to be respectively laevo- and dextro-rotatory. The dextro-acid seemed to show less tendency to become coloured; solutions in alcohol, left exposed to the light for six weeks in stoppered bottles, remained colourless.

The laevo-specimen of the acid was reduced with platinum black and hydrogen in ether solution and a compound obtained which melted after several recrystallisations at 165°–167°, and had $[\alpha_D] = - 88·1°$. This substance is probably similar to that obtained by Johanssohn [1917] by reducing under similar conditions a specimen of abietic acid which melted from 168°–173° and had $[\alpha_D] = - 28·6°$. His reduced acid softened at 156° and melted from 173°–178°, the rotation not being given. In our case the specific rotation of the acid was raised by reduction from − 73·5° to − 88°.

*Iodine values.*

Fahrion [1901], Vesterberg [1907] and Johanssohn [1917] examined the iodine values of these acids using the Hübl-Waller method. Johanssohn found considerable variations according to the concentration and time of contact

with the iodine solution but made the curious observation that the iodine value of the reduced acid was practically identical with that of the original acid and showed this was also true for pimaric and dihydropimaric acid.

If abietic acid and its dihydro-derivative are examined by the Hübl and Wijs methods almost identical values are obtained with the latter reagent but the Hübl solution differentiates between them, the value for the dihydro-acid having been reduced by the amount required by one double bond.

| | Hübl-Waller iodine value | No. I atoms added per mol. substance | Hübl iodine value | No. I atoms added per mol. substance | Wij's iodine value | No. I atoms added per mol. substance |
|---|---|---|---|---|---|---|
| Abietic acid ... | 173·9[1] 164[2] | 4 | 162 | 4 | 239 | 6 |
| Dihydro-abietic acid | 166[2] | 4 | 75 | 2 | 232 | 6 |
| Cholesterol ... ... | — | — | 72 | 2·3 | 158 | 5 |

[1] Fahrion. [2] Johanssohn.

If the difference between the Hübl and Wijs values represents the absorption due to substitution, in dihydro-abietic acid twice as much substitution takes place as in abietic acid itself. In dihydro-abietic acid as in cholesterol the presence of a condensed ring nucleus containing one double bond seems to exert a considerable influence. In dihydro-abietic acid the nucleus has almost certainly a reduced phenanthrene structure.

The main points on which a formula for abietic acid [cp. Levy, 1913] may be based are:

(1) Analysis and neutralisation values agree best with the formula $C_{20}H_{30}O_2$ [Levy, 1905].

(2) The COOH group present is not esterified by the action of alcohol and HCl; therefore there are probably two substituents in the ortho-position to the carboxyl groups [Easterfield and Bagley, 1904].

(3) By oxidation with nitric acid, hexahydrophthalic acid has been obtained, pointing to the presence of a terminal hexahydrobenzene ring without substituents [Levy, 1905].

(4) On distilling abietic acid with sulphur, retene (1-methyl-7-*iso*propyl-phenanthrene) is obtained [Vesterberg, 1903], so that a phenanthrene nucleus is probably present in abietic acid, or else a group which will readily pass into phenanthrene [Levy, 1913].

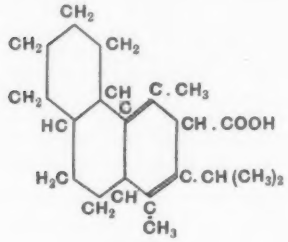
If a terminal hexahydrobenzene nucleus be present in the abietic acid molecule, the wandering of a methyl group during the distillation must be assumed.

(5) An *iso*propyl group is present, since *iso*butyric acid was obtained from the products of oxidation with permanganate [Levy, 1906].

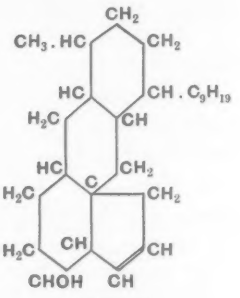
(6) Four halogen atoms are absorbed from Hübl's solution, agreeing with presence of two double bonds, neither being in the $\alpha$, $\beta$-position to the COOH group.

(7) Reduction at ordinary temperature in ethereal solution with hydrogen in the presence of platinum black leads to the addition of two hydrogen atoms [Johanssohn, 1917]; the dihydro-abietic acid thus formed contains one double bond in the molecule, since it still reacts with both iodine solutions.

Neither the position of the double bonds nor that of the alkyl substituents in abietic acid is definitely established, but the above facts are represented by some such formula as the following:




Abietic acid




Cholesterol

The most recent formula ascribed by Windaus [1920] to cholesterol is added for the purpose of comparison.

## Conclusions.

1. When iodine values are determined by both the Wijs and the Hübl methods the Hübl iodine values obtained are much higher than the Wijs in the case of phenol and to a less extent of $\alpha$-naphthol. Since the Wijs reagent acts as an enolising agent, a possible explanation is that a change to keto-dihydrobenzene precedes the addition of halogen and that this change is retarded by the Wijs reagent [cp. Ingle, 1904].

2. The Wijs iodine numbers are much higher than the Hübl values in the case of four sets of compounds amongst those examined: (*a*) fatty carbonyl compounds, (*b*) the phenanthrene hydrocarbons, (*c*) sterols and (*d*) the resins.

3. It is suggested that, in accordance with Lapworth's work, the carbonyl compounds are enolised by the acid of the Wijs reagent and that the addition of halogen then takes place. This enolising action is brought about only very slightly by the neutral Hübl reagent.

In carbonyl groups with conjugated ethenoid groups, where enolisation does not so readily occur, the Hübl values are equal to or greater than those given by the Wijs reagent.

4. Anthracene absorbs from 2 to 4 halogen atoms and does not differentiate between the Hübl and Wijs reagents. Phenanthrene absorbs up to 1·31 atoms

from the Wijs solution but is almost without action on the Hübl. The Wijs value depends largely on temperature and the relative iodine concentration.

5. The conversion of anthracene and phenanthrene to the corresponding quinones completely prevents halogenation by the iodine reagents.

6. Abietic acid reacts with Hübl and Wijs solutions, the iodine values obtained corresponding to the absorption of approximately 4 and 6 atoms of iodine respectively. The Hübl value indicates that two double bonds are present in the abietic acid molecule, which is in accordance with what we know of its constitution.

7. Addition of two hydrogen atoms to the abietic acid molecule leaves the Wijs value unchanged but reduces the Hübl value to that indicating the presence of one double bond in the dihydro-abietic acid formed.

8. The Hübl values of the sterols are variable but indicate approximately the number of double bonds believed to be present in the molecule. The Wijs numbers are very much greater and probably indicate that a considerable amount of substitution has taken place. The reduced sterols show small iodine values whether examined by the Hübl or Wijs reagent.

9. In the sterols and resins the presence of a condensed ring nucleus containing one or two double bonds influences the amount of substitution produced by the Wijs reagent. The actual number of double bonds present is approximately indicated if the iodine value be determined by Hübl's solution.

10. In any fat containing appreciable quantities of sterol, the Hübl iodine value more accurately represents the degree of unsaturation of the mixture than does the Wijs value. The Wijs values of many fats are appreciably higher than the Hübl numbers [Wijs, 1898]. This difference is significant and indicates the presence of sterol in the fat.

This investigation has arisen out of work carried out for the Food Investigation Board of the Department of Scientific and Industrial Research and we gratefully acknowledge the grants provided for this purpose.

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# XXXVI. THE EFFECT OF EXPOSURE TO LOW TEMPERATURES ON SOME PHYSIOLOGICAL, CHEMICAL AND PHYSICAL PROPERTIES OF AMPHIBIAN MUSCLE.

By DOROTHY LILIAN FOSTER and DOROTHY MARY MOYLE.

*From the Biochemical Laboratory, Cambridge.*

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Fatigue in muscle is a phenomenon which has excited the interest of observers ever since it was discovered that an excised amphibian muscle would respond to an electric stimulus by a contraction, but that if the stimuli were continued long enough the response grew weaker, and finally ceased, however strong the stimulus. The muscle was then obviously in a condition similar to that of fatigue in the living animal. The nature of this state of fatigue has been the subject of much research, but the work of Fletcher and Hopkins [1907] was by far the most important. They showed that fatigue is accompanied by the accumulation of lactic acid in the muscle, as is also rigor mortis, however that rigor may have been produced. Since loss of irritability is an invariable accompaniment of rigor, whether in fatigue or in the more complete and irreversible, but similar, state of death, it has been usual to regard this inability to respond to stimuli as due to the accumulation of acid. It seems that, although the equilibrium between a complicated set of factors necessary for irritability is maintained for some time after excision, yet, in the absence of the circulation, the accumulation of products of metabolism, notably lactic acid, finally upsets it. As is described in the following pages, however, it has now been shown that a non-irritable condition can exist without accumulation of lactic acid; it is, therefore, necessary to look upon loss of irritability as a separate phenomenon from the rigor of fatigue and death. Workers with amphibian muscle have occasionally noted that their material was non-irritable, but not rigid, although they have not attributed any significance to this fact, or made any determinations of the acid content of the muscle. Fletcher [1902] showed that muscle kept in oxygen did not become rigid, and Fletcher and Hopkins [1907] noted that their muscle was no longer irritable, though the condition was not one of fatigue.

Obviously, in the non-irritable condition described in the present paper, there is involved another factor, which, under ordinary circumstances of fatigue and death rigor, either does not come into play at all (possibly because

time is necessary for its action) or is masked by the more obvious factor of lactic acid accumulation.

Up to the present we have investigated the effect of prolonged exposure to 0° C. on the irritability, on the power of lactic acid formation under different conditions, and on the osmotic properties of amphibian muscle.

## Methods.

Before adopting a routine method of lactic acid estimation a comparison was made between the method of Fletcher and Hopkins [1907] and that more recently advocated by Wolf [1914]. It was found that Wolf's method had no advantage over the other as regards saving of time, and, in our hands at any rate, it did not give the more exact results claimed for it. Moreover, it is not applicable in all cases; for example, in the determination of the resting minimum.

Hence it was decided to adopt in all details the familiar method of the earlier investigators. At the beginning of this work it was not possible to obtain Merck's charcoal, but it was found that the finest grade of birchwood charcoal, when thoroughly washed, could be used instead. Otherwise, we followed in all respects the methods of extraction and estimation described in the above-mentioned paper, so that further description here is unnecessary.

One other point may be mentioned in connection with methods. Mondschein [1912] states that lactic acid results are usually too low, because a large proportion, as much as 33 % of the acid, remains in combination with the protein, from which it can only be set free by alkaline hydrolysis. He, however, used extraction with boiling water as his first stage, and it was felt that his results could not apply to extraction with ice-cold alcohol. In order to test this, the residue, after extraction with alcohol in the case of both frog muscle and beef, was hydrolysed with strong alkali as described by Mondschein, and his procedure followed in detail; but only traces of lactic acid were recovered by this means, and in some cases none at all. It was therefore decided that, as alcohol was used for all extractions, this further treatment was unnecessary.

In studying the effect of exposure to continuous low temperatures on the lactic acid production, it was decided to work at 0° C. because a temperature which is sufficiently low to freeze the muscle fluids will cause rupture of the fibres, and this in itself might be expected to be sufficient to lead to a large production of lactic acid. The method adopted was the following.

The frogs were pithed and the hind limbs skinned as carefully as possible, and then severed above the girdle. The limb pairs were then arranged on long glass slides, about ten or twenty on a slide; each slide fitted into a glass tube about 2½ feet long, and 2½ inches wide. A little Ringer's solution was run in under the slide, and the tube then closed by rubber stoppers, carrying glass leading tubes. The end limb pair was used as a test, and not included in the subsequent estimations. Thin copper wires were twisted round the legs of

this pair, and then passed up one of the leading tubes, to the terminals of an induction coil. The primary circuit consisted of an accumulator (1·4 volts) and key. When the limbs were exposed to atmospheres of oxygen or nitrogen, one stopper had a second leading tube which could be attached, *via* the necessary bubblers, to the cylinder of the gas in question. The tube at the opposite end was attached to a bubbler, so that it could be ascertained whether a steady flow of gas was being maintained. Three or four tubes, according to the number of limb pairs required, were tied to a board, which was then placed in a trough in a sink, and the whole kept filled with crushed ice as long as the experiment lasted. Special arrangements were made to allow for drainage. The whole procedure, from the pithing of the first frog to the covering with ice, generally took ten minutes.

I. The effect of exposure to 0° C. on the irritability of frog muscle.

The irritability was tested twice a day by make and break induction shocks, the coil being moved out until the maximum distance (minimum shock) was found which would produce a response at make and break respectively. By plotting time against position of the coil, a curve of declining irritability was obtained. Such a record was kept for two experiments, but in all later ones, when the approximate time was known, the limbs were left for a week before being tested at all. The duration of irritability varied with the time of the year and the condition of the frogs, but the minimum period, in air or oxygen, was a week[1]. An interesting observation, made by other workers, was confirmed; namely, that the muscles cease to be irritable in a definite order, beginning with those of the thigh, and ending with those of the toes.

The muscles rendered non-irritable in air or oxygen in this way were quite flaccid and clear, showing no trace of the stiffness and opacity familiar in muscles which have been kept in air at higher temperatures, and which appeared at this low temperature in muscles kept in nitrogen.

The curves obtained for air and oxygen atmospheres were very characteristic. There is a rapid falling off of irritability, followed by a period of little change, and again a rapid fall to non-irritability. The meaning of these stages is not yet clear. The curves for nitrogen atmosphere show a rapid fall only. The curves for one experiment are appended.

Fig. 1 shows the curve for winter frogs, which remain irritable longer than spring frogs. Note the marked difference in effect between air and nitrogen. The lactic acid determinations confirm this in a striking way.

II. A. The effect of exposure to 0° C. on the "survival" production of lactic acid in frogs.

When it was found that muscles could be rendered non-irritable simply by exposing them to a temperature of 0° C., it was decided to investigate the survival production of lactic acid under these conditions, in order to see

[1] The limb pair used as test always lost irritability sooner than the others, presumably owing to the poisonous action of the copper.

whether the physiological change was accompanied by an alteration in the chemical properties of the muscle.

When the resting minimum lactic acid was estimated in the non-irritable muscles of Experiment I, it was found to be strictly comparable with that of fresh untreated muscle, *i.e.* 0·01–0·03 %. This means that there was no measurable formation of lactic acid in the muscles kept at 0°, either in oxygen or in air. When normal, fresh muscle is maintained in air at room temperature, there is a slow steady production of lactic acid, equal increments being formed in equal lengths of time, as was shown by Fletcher and Hopkins. As a control for Experiment I, ten limb pairs were placed, with all precautions against infection, in sterile Petri dishes, and kept at room temperature for 53 hours. At the end of that time there was not the slightest trace of a putrefactive smell and all limbs were stiff (Expt. VIII). These muscles contained 0·112 % lactic acid as compared with the probable 0·02 % of fresh muscle. As is well

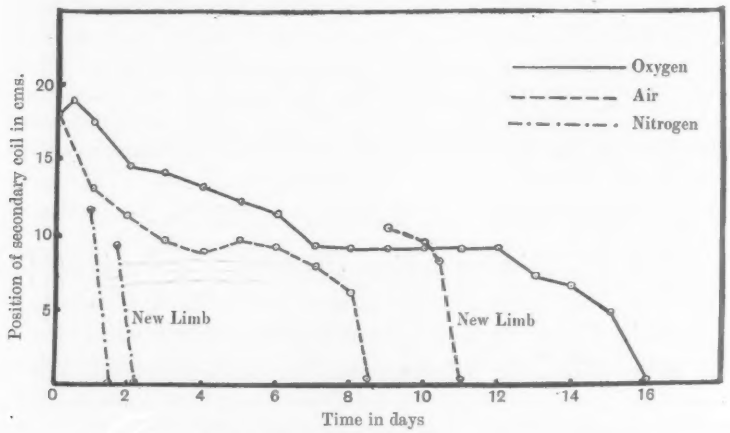


Fig. 1. Curve of declining irritability at 0° C. Break shock record.

known, on the other hand, in an atmosphere of oxygen, there is no measurable accumulation of lactic acid upon survival at room temperature [Fletcher and Hopkins, 1907].

This experiment on non-irritable muscles has been repeated several times, and always with the same result, that prolonged exposure to a temperature of 0° produces non-irritability without increase of lactic acid content, whether the muscle has been exposed to air or pure oxygen. That is to say, at this low temperature, the tension of oxygen in air is as effective as that of pure oxygen at higher temperatures. The following experiments were made, the details being given in the table (p. 341), together with figures quoted from Fletcher and Hopkins [1907] for comparison.

*Experiment I.* Muscles kept at 0° till non-irritable.

(*a*) In air. Lactic acid, 0·017 %.

(*b*) In oxygen. (1) Lactic acid, 0·021 %; (2) lactic acid, 0·023 %.

*Experiment II.* The effect of different atmospheres at 0°. Muscles kept at 0° till non-irritable. Survival lactic acid.

(*a*) In air. Lactic acid, 0·042 %. (The high result in this case was due to experimental error—the final residue not being pure.)

(*b*) In oxygen. Lactic acid, 0·017 %.

At first it was thought that the muscle might be incapable of forming lactic acid at this low temperature, but this was shown not to be the case as soon as we exposed the muscle to an atmosphere of nitrogen. Then we found the survival production of lactic acid, so characteristic of anaerobic conditions at normal temperatures. The rate of production is delayed, it is true, but this is only to be expected, and the "flattening of the curve" produced by a fall in temperature from 18° to 12° was shown by Fletcher and Hopkins [1907].

Hence this non-production of lactic acid on survival at 0° is not due to the inability of the muscles to form the acid, but probably due to the slowing down at that temperature of the processes which result in the production of lactic acid, so that the tension of oxygen in atmospheric air is sufficient to remove it as fast as it is formed and the conditions are equivalent to those of normal muscle in oxygen at higher temperatures.

(*c*) (1) Nine limb pairs kept 48 hours in nitrogen at 0°. Lactic acid, 0·130 %.

(2) Ten limb pairs kept six days in nitrogen at 0°. Lactic acid, 0·248 %.

### II. B. The chemical properties of non-irritable muscle.

In the preceding section it is shown that it is possible to avoid the survival production of lactic acid in frog's muscle by exposure to 0° till irritability is lost. It now remained to see if it is possible for lactic acid to be produced under any circumstances in the non-irritable muscle.

We first tried the effect of keeping the muscle at room temperature for 24 hours, and found only a very trifling increase in lactic acid. Whether more would be formed if it were possible to keep the muscle longer at room temperature is not known, as even at the end of 24 hours putrefaction is just beginning.

*Experiment I.*

(*c*) Muscles rendered non-irritable by exposure for 12 days to a temperature of 0° in air, and then left overnight at room temperature. Lactic acid, 0·042 %.

*Experiment III.*

(*a*) Ten limb pairs rendered non-irritable by keeping at 0° in air for eight days. Then left for 24 hours at room temperature. Lactic acid, 0·036 %.

In one case the muscles were left in ice for four days after all signs of irritability had disappeared, and it was found that there was no increase of lactic acid.

*Experiment II.*

(*d*) Muscles kept at 0° in air for four days after loss of irritability. Lactic acid, 0·035 %.

It has been shown above that muscles kept in nitrogen at 0° slowly develop lactic acid. It remained now to see whether muscle rendered non-irritable by keeping at 0°, would produce lactic acid if kept in nitrogen for a further period, at the same temperature. It was found that the non-irritable muscle behaved exactly like a fresh untreated muscle, in that, in the absence of oxygen, there was a production of lactic acid. A characteristic maximum was not reached, but any survival processes which do persist must be very considerably slowed at 0°, and hence a maximum was hardly to be expected in the time allowed.

*Experiment V.*

Ten limb pairs were rendered non-irritable by exposure, in air, to a temperature of 0° for seven days. The tube was then cleaned, the limbs replaced, and a current of nitrogen passed through the tube continuously for five days. At the end of that time the muscles were slightly opaque, but did not show much rigidity. Lactic acid, 0·148 %.

The non-irritable muscle was next subjected to those conditions which, in fresh muscle, cause maximum production of lactic acid; namely, heat, mechanical injury and the action of certain substances, *e.g.* toluene and chloroform. It was found that, in these circumstances, the muscle behaved in no way differently from normal fresh muscle, a maximum being produced in each case.

*Experiment II.*

(*e*) Heat rigor in non-irritable muscle.

The limbs, after removal from the ice, were spread on the bottom of thin-walled, covered beakers sunk in a thermostat at 45°, and maintained at this temperature for 1½ hours. This treatment produced the characteristic condition of stiffness and opacity associated with heat rigor. Lactic acid, 0·229 %. (N.B. These were summer frogs, which would account for the rather low maximum.)

(*f*) Effect of toluene on non-irritable muscle.

The tube, after removal from the ice, was placed on the bench at room temperature, and the ends plugged with cotton wool, soaked in toluene. Lactic acid, 0·261 %.

*Experiment IV.*

(*a*) Effect of toluene (repeated).

In this experiment the tube containing the non-irritable limbs was connected at one end to the pump, and at the other to a flask containing toluene;

so that a continuous current of toluene vapour could be drawn through the tube for 24 hours. Lactic acid, 0·185 %.

In this same experiment a series of non-irritable muscles was exposed to toluene at 0° with the following result.

(*b*) Effect of toluene on non-irritable muscle at 0°.

Lactic acid, 0·024 %.

The effect of injury, both at room temperature and at 0°, was next investigated; in the latter case it was found that the production of the lactic acid maximum was delayed.

*Experiment I.*

(*d*) Effect of injury.

The muscle was dissected from the limbs after removal from the ice, put through a mincer and finally rubbed to a paste in a mortar. It was then allowed to stand at room temperature over night to make sure that sufficient time had elapsed for a maximum production of acid [Fletcher, 1911]. Lactic acid, 0·291 %.

*Experiment II.*

(*g*) Effect of injury at 0°.

The non-irritable muscle was minced at 0°, and left at 0° over night. Lactic acid, 0·218 %.

An interesting confirmation of the effect of injury was obtained by the action of extreme cold. The freezing point of amphibian muscle is − 0·47°, and at this temperature ice crystals separate. At still lower temperatures the whole muscle freezes solid. This means a rupture of the muscle fibres and is equivalent to mechanical injury. Fletcher showed that severe freezing and subsequent thawing was analogous to mechanical injury, in that it resulted in the production of lactic acid [1914]. This was confirmed by us, and further it was found that freezing non-irritable muscle to a temperature of − 8° resulted in the formation of lactic acid. In the cases of both fresh and non-irritable muscle, lactic acid was found to be present, both when the muscle was thawed before estimation, and when the temperature during extraction was not allowed to rise above 0°.

*Experiment VI.*

(*a*) Effect of cold storage on non-irritable muscle.

A series of frog's limbs were rendered non-irritable by exposure to 0° for two weeks, and then placed in cold storage at − 8° for three weeks. Every care was taken to prevent rise of temperature until the end of the extraction. Lactic acid, 0·163 %.

(*b*) Effect of cold storage and subsequent thawing.

Seven of the frozen limb pairs, obtained as above, were allowed to thaw before being extracted. Lactic acid, 0·174 %.

Summary of experimental results

| No. of expt. | Nature of experiment | Special treatment | State of muscle | % lactic acid |
|---|---|---|---|---|
| I (*b*) | The effect of various atmospheres | Oxygen 18 days at 0° | Non-rigid, non-irritable | 0·021 |
| II (*b*) | ,, | ,, | ,, | 0·023 |
| I (*a*) | ,, | Air 12 days at 0° | ,, | 0·017 |
| II (*a*) | ,, | — | — | 0·042 |
| II (*c*) (1) | ,, | Nitrogen 2 days at 0° | Non-irritable incipient rigidity | 0·130 |
| II (*c*) (2) | ,, | 5 days at 0° | Non-irritable and rigid | 0·248 |
| II (*e*) | Chemical properties | | | |
| | Heat | 7 days at 0° 1½ hrs. at 45° | Non-irritable | 0·229 |
| II (*f*) | Toluene | 7 days at 0° + 24 hrs. exposure to toluene at 22·5° | Non-irritable | 0·261 |
| IV (*a*) | | do. | Non-irritable | 0·185 |
| I (*d*) | Injury | 12 days at 0° minced and left over night at room temp. | Non-rigid, non-irritable | 0·291 |
| II (*g*) | | 12 days at 0° and minced and left at 0° over night | ,, | 0·218 |
| V | Nitrogen | 7 days in air at 0° followed by exposure to N at 0° 5 days | Non-irritable | 0·148 |
| I (*c*) | Other properties of non-irritable muscle | | | |
| | Thawing | Muscle at 0° in air 12 days, at room temp. over night | Non-irritable and non-rigid | 0·042 |
| VI (*a*) | Freezing | Muscle at 0° 15 days, −8° 3 weeks | Non-irritable when frozen | 0·163 |
| II (*d*) | Prolonged exposure to 0° | Muscle at 0° 7 days till non-irritable and then 4 days more | Non-irritable | 0·035 |

Controls at higher temperature

| Expt. | Treatment | % lactic acid | State of muscle | Reference |
|---|---|---|---|---|
| VIII | Oxygen at 13° 23 hrs. | 0·028 | — | F. and H. |
| VIII | Air at 15° 53 hrs. | 0·083 | — | F. and M. |
| X | Hydrogen at 16° 35½ hrs. | 0·148 | Non-irritable more or less rigid | F. and H. |
| V | Heat at 45° | 0·231 | — | F. and H. |
| XX | Chloroform at 20° | 0·329 | — | F. and H. |
| I | Minced and left in hydrogen at 15° | 0·399 | — | F. and H. |
| II | Minced and left in hydrogen at 10° | 0·197 | — | F. and H. |
| VII (*a*) | Fresh muscle at −8° 4½ hrs. | 0·085 | — | F. and M. |
| VII (*b*) | Fresh muscle 65 days at −8° | 0·188 | — | F. and M. |

F. and H. = Fletcher and Hopkins [1907].
F. and M. = present authors.

so that a continuous current of toluene vapour could be drawn through the tube for 24 hours. Lactic acid, 0·185 %.

In this same experiment a series of non-irritable muscles was exposed to toluene at 0° with the following result.

(*b*) Effect of toluene on non-irritable muscle at 0°.

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A series of frog's limbs were rendered non-irritable by exposure to 0° for two weeks, and then placed in cold storage at − 8° for three weeks. Every care was taken to prevent rise of temperature until the end of the extraction. Lactic acid, 0·163 %.

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| V | Nitrogen | 7 days in air at 0° followed by exposure to N at 0° 5 days | Non-irritable | 0·148 |
| I (*c*) | Other properties of non-irritable muscle | | | |
| | Thawing | Muscle at 0° in air 12 days, at room temp. over night | Non-irritable and non-rigid | 0·042 |
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Controls at higher temperature

| Expt. | Treatment | % lactic acid | State of muscle | Reference |
|---|---|---|---|---|
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| V | Heat at 45° | 0·231 | — | F. and H. |
| XX | Chloroform at 20° | 0·329 | — | F. and H. |
| I | Minced and left in hydrogen at 15° | 0·399 | — | F. and H. |
| II | Minced and left in hydrogen at 10° | 0·197 | — | F. and H. |
| VII (*a*) | Fresh muscle at −8° 4½ hrs. | 0·085 | — | F. and M. |
| VII (*b*) | Fresh muscle 65 days at −8° | 0·188 | — | F. and M. |

F. and H. = Fletcher and Hopkins [1907].
F. and M. = present authors.

*Experiment VII.* The effect of cold storage on fresh muscle.

(*a*) Ten limb pairs were placed in cold store at $-8°$ for $4\frac{1}{2}$ hours. When removed they were frozen hard, and the temperature was not allowed to rise above 0° during the extraction. Lactic acid, 0·085 %.

(*b*) Ten limb pairs were left in cold store for 65 days, and then extracted with the same precautions as before. Lactic acid, 0·188 %.

### Summary of Parts I and II.

It is shown that by continuous exposure to a temperature of 0° it is possible to produce a condition in frog muscles in which they are non-irritable but at the same time non-rigid and to all outward appearance similar to normal fresh muscle.

This loss of irritability is reached without the survival production of lactic acid if the muscle has been kept in air or oxygen. In nitrogen, however, the loss of irritability is much more rapid and is attended with formation of lactic acid. In nitrogen, too, the muscles become rigid and opaque.

Certain chemical properties of the muscle usually associated with irritability and the act of contraction were then investigated to see if the loss of this physiological property could be correlated with any chemical manifestation in the muscle. No such correlation was found. Apart from the absence of "survival" lactic acid the non-irritable muscle formed lactic acid in exactly the same circumstances as fresh irritable muscle. The explanation of this condition in the muscle was then sought in its physical properties, and Part III deals with this side of the investigation.

## III. Physical properties of the non-irritable muscles.

As has been described in Part I of this report, the non-irritable muscles obtained by the prolonged action of a temperature of 0° were found, as regards the chemical property usually believed to be most intimately connected with changes in irritability, *i.e.* lactic acid content, to be practically identical with fresh, irritable muscle. It was therefore necessary to look for changes in the physical properties of the muscle. For several reasons, possible alterations in the permeability of the semi-permeable membranes appeared worthy of first attention.

According to the theory of excitation put forward by Keith Lucas [1910], the special condition for excitation is the accumulation of certain ions in the neighbourhood of a membrane. This accumulation is caused by the fact that the membrane is impermeable to these ions, and it is therefore possible that an increase of permeability of the membrane would abolish excitability. Again, a possible explanation of the fact that in the non-irritable muscle left for 24 hours at room temperature no lactic acid is formed, would be a change in the permeability of some membrane preventing the coming together of enzyme and substrate.

As the conductivity method of measuring permeability was difficult, owing to the stimulating action of the electric current on the normal control muscle, the osmotic method was used. It should be noted that the results obtained by both these methods are capable of two interpretations. For instance, diminished conductivity and osmotic pressure may both be caused *either* by increased permeability of the membrane, *or* by decrease in the number of conducting and osmotically active particles. This latter factor may play an important part. For instance, it was suggested to us by Professor Hopkins that, during the long standing of the muscle at 0°, it is likely, from a physico-chemical point of view, that the colloids may adsorb dissolved electrolytes, thus fixing them and making them incapable of conduction or osmotic activity. The presence of these ions on the membrane might cause a change in its permeability, and might also result, according to Lucas' hypothesis, in a loss of excitability, owing to the non-mobility of the ions.

We also thought that it might be instructive to be able to compare the osmotic properties of these special non-irritable muscles with the osmotic properties found by Fletcher [1903] for muscle which had more or less lost excitability (muscle in death rigor, and fatigued muscle).

*Method.*

The non-irritable muscles were obtained in the usual way.

Nine gastrocnemii were removed as carefully as possible, and placed in a covered Petri dish on ice. Loops of cotton were tied to the tendons, and the muscles then weighed, in sets of three, in tared, covered watch glasses. Eight gastrocnemii from freshly pithed frogs were similarly treated as controls. The muscles were then suspended in different solutions by hanging the cotton loops on wire hooks, which passed through the corks of the conical flasks containing the solutions.

The following solutions were used:

| | | | |
|---|---|---|---|
| Flask 1. | 0·2 % NaCl | 3 non-irritable muscles. | |
| 2. | 0·75 % NaCl | ,, | ,, |
| 3. | 7·5 % cane sugar | ,, | ,, |
| 4. | 0·2 % NaCl | Control. | 3 muscles. |
| 5. | 7·5 % cane sugar | ,, | 3 ,, |
| 6. | 0·75 % NaCl | ,, | 2 ,, |

Each flask contained 600 cc. of solution.

The sodium chloride solutions are of the same strengths as those used by Fletcher [1903], the 0·2 % being hypotonic for normal muscle, and the 0·75 % isotonic at 17°. The 7·5 % cane sugar is, according to Meigs [1919], isotonic.

The sets of muscles were removed from the solutions about once every 1½ hours and weighed, the time being noted. The cotton loops were thoroughly blotted with filter paper, and the muscles dried by drawing across filter paper two or three times. This procedure was made as uniform as possible.

The weighings were repeated at regular intervals for about 12 hours and then a final weighing was obtained at the end of 24 hours.

This procedure was followed in Experiments X and XI with certain additions. The effect of distilled water, as well as that of the isotonic and hypotonic solutions already described, was investigated. It should be pointed out that the muscles used in Experiments IX and X were from summer frogs and those of Experiment XI were from winter frogs. This would account for individual variations [see Fletcher, 1903].

The curves from three experiments are appended (Figs. 2, 3 and 4).

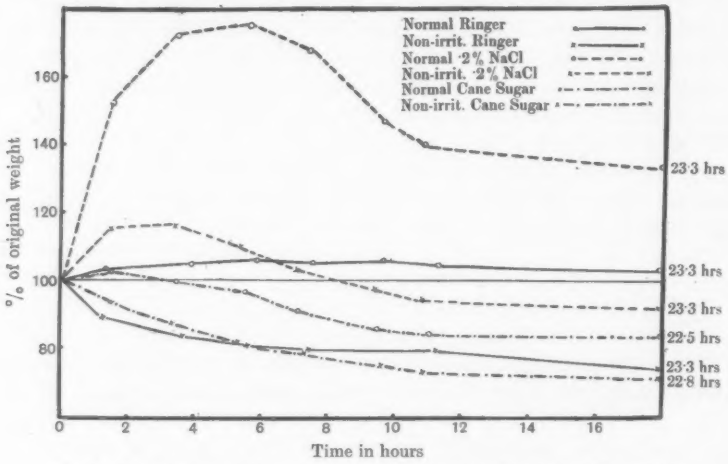


Fig. 2. Expt. IX. The osmotic properties of normal and non-irritable muscle.

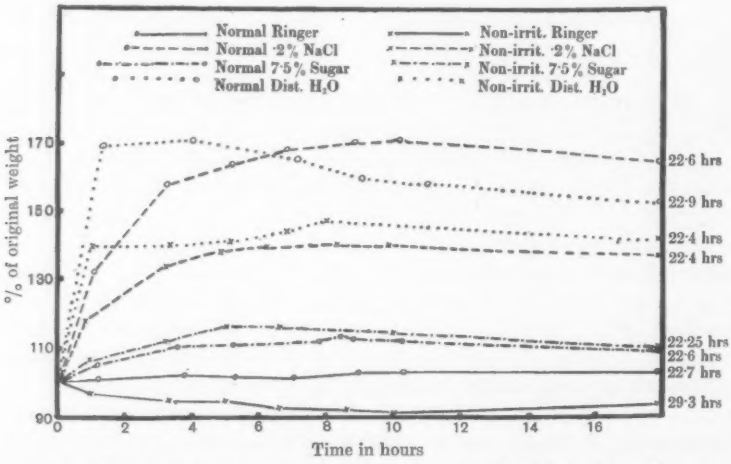


Fig. 3. Expt. X. The osmotic properties of normal and non-irritable muscle.

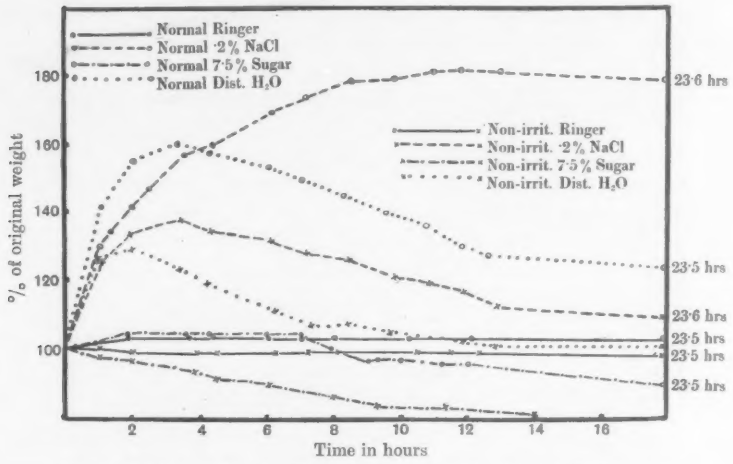


Fig. 4. Expt. XI. The osmotic properties of normal and non-irritable muscle.

## Results.

One property illustrative of the condition of the non-irritable muscle is clearly shown in all these curves. The osmotic condition is such that a solution which is isotonic to a normal muscle is hypertonic to the non-irritable muscle and correspondingly a solution which is hypotonic to a normal muscle is less hypotonic to the non-irritable muscle. (N.B. The isotonic cane sugar in Experiment X would seem to be an exception, but it is well known that cane sugar has special effects on muscle [see Bayliss, 1918].)

If these curves are compared with those given by Fletcher [1903] for fatigued muscle, it will be seen at once that, though differing from the normal, they are in no way representative of those for fatigued muscle. As already suggested this osmotic behaviour of the non-irritable muscle can be accounted for in two ways:

(*a*) The membrane may have become more permeable.

(*b*) There may be a diminution in the number of osmotically active particles—as for instance by adsorption on to the surface of colloids.

Further work must attempt to discriminate between these alternatives. By the kindness of Dr Adrian we have been able to ascertain that the electrical properties show a variation from the normal. It was found that a non-irritable sartorius gave no current of action on stimulation, but there was a demarcation current which was, however, considerably less than the normal.

Thus we have found well-marked physical, though no chemical, differences in the non-irritable amphibian muscle.

More recently we have found that, by treating the non-irritable muscle with various solutions, it is possible to recover a certain degree of irritability.

An amphibian "Ringer" solution, made according to Bayliss' formula, was most efficient but was almost equalled by an isotonic solution of pure sodium chloride. The interval before recovery was very short in these solutions but was unaffected either by exposure to oxygen or to nitrogen atmospheres. This lends support to the view that the loss of irritability is of "ionic" origin. Up to the present it has only been possible to get a slight recovery; the twitch is small and the stimulus required very large. Work on these lines is still proceeding and will form the subject of a future communication.

We wish to express our great indebtedness to Professor Hopkins who proposed this work to us and who has made many valuable suggestions throughout.

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